Name	Sequence	Primer/Probe Type	Paper
ISA8			
ISAV-FS8	CGACGATGACTCTCTACTGTGTGAT	primer	Plarre H, Devold M, Snow, M, Nylund A (2005). Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in Western Norway. Dis Aquat Org 66:71 – 79
ISAV-RS7	GAAAATCCATGTTCTCAGATGCAA	primer	
ISAV-S8	6FAM-ACGGTGGATCTTTC	MGB probe	
ISA7			
ISAV-FS7	TGGGATCATGTGTTTCCTGCTA	primer	Plarre H, Devold M, Snow, M, Nylund A (2005). Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in Western Norway. Dis Aquat Org 66:71 – 79
ISAV-RS8	TCATCAGTGTCGCCATGCTT	primer	
ISAV-S7	6FAM-CACATGACCCCTCGTC	MGB probe	
ISA8_2010			
ISAs8-1F-2010	CGAAAGCCCTGGAAACTTTAGA	primer	Debes H. Christiansen etc. (2010) A low-pathogenic variant of infectious salmon anemia virus (ISAV-HPRo) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (Salmo salar L.) in the Faroe Islands. J Gen Virol Vol.92 no. 4 909-918.
ISAs8-1R-2010	GATGCCGGAAGTCGATGAACT	primer	
ISAs8-P-2010	5'-6FAM-AAGGCCATCGTCGCT	MGB probe	
ISA_snow7			SNOW M., MCKAY P., MCBEATH A. J. A., BLACK J., DOIG F., KERR R., CUNNINGHAM C. O., NYLUND A. & DEVOLD M. (2006). Development, application and validation of a taqman® real-time RT-PCR assay for the detection of infectious salmon anaemia virus(ISAV) in Atlantic salmon (Salmo salar), Vannier P. & Espeseth D., eds. New Diagnostic Technology: Applications in Animal Health and Biologics Controls. Dev. Biol., Basel, Karger. 126, 133–145.
ISAV-snow7_F	5'- CAGGGTTGTATCCATGGTTGAAATG	primer	
ISAV-snow7_R	5'- GTCCAGCCCTAAGCTCAACTC -3'	primer	
ISAV-snow7_P	5'-6FAM- CTCTCTCATTGTGATCCC	MGB probe	
ISA_snow8			
ISAV-snow8_F	5'- CTACACAGCAGGATGCAGATGT -3'	primer	
ISAV-snow8_R	5'- CAGGATGCCGGAAGTCGAT -3'	primer	
ISAV-snow8_P	5'-6FAM- CATCGTCGCTGCAGTTC	MGB probe	

Sequencher - [ISA8]

File   Edit   Select   Contig   Sequence   View   Window   Help

Bases   Summary   Sort   Options   Find   Get Info

Marty's primers

ISA8_Gary

Molly Kibenge's
sequences from
2003 and 2004
(raw data on
server)
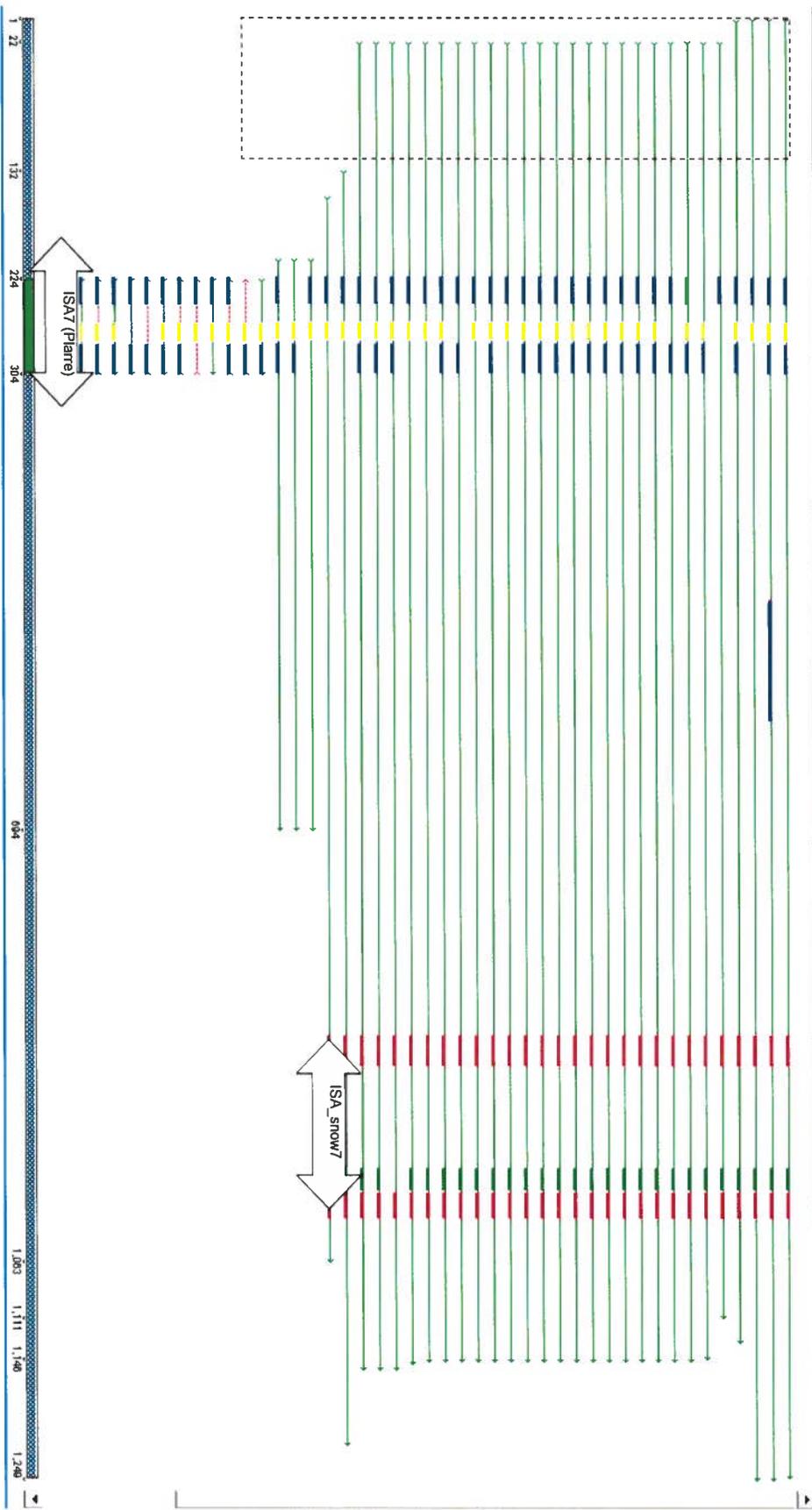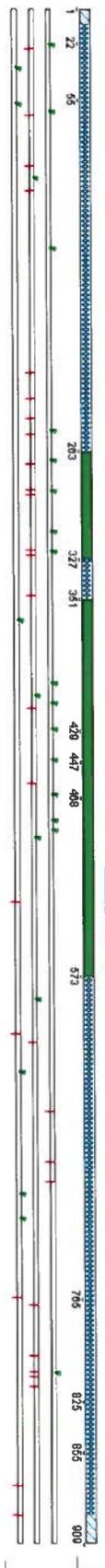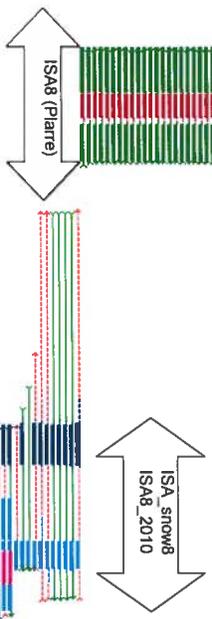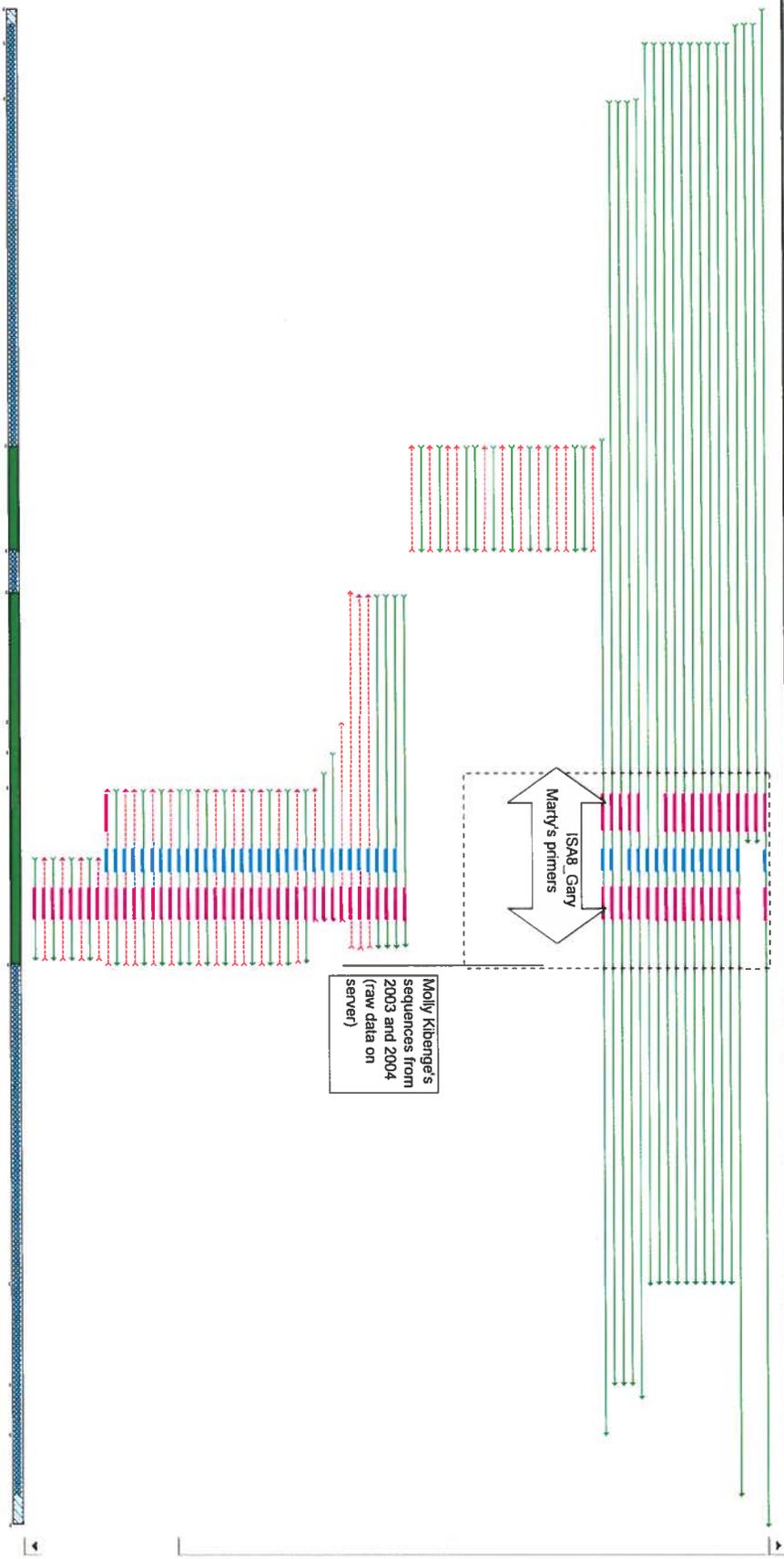
NOTE

Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway

H. Plarre[1], M. Devold[1], M. Snow[2], A. Nylund[1,*]

[1]Department of Biology, University of Bergen, PO Box, 5020 Bergen, Norway
[2]FRS Marine Laboratory, PO Box 101, Victoria Road, Torry, Aberdeen AB11 9DB, UK

ABSTRACT: Studies of infectious salmon anaemia virus (ISAV), an important pathogen of farmed salmon in Norway, Scotland, the Faeroe Islands, Ireland, Canada, the USA and Chile, suggest that natural reservoirs for this virus can be found on both sides of the North Atlantic. Based on existing information about ISAV it is believed to be maintained in wild populations of trout and salmon in Europe. It has further been suggested that ISAV is transmitted between wild hosts, mainly during their freshwater spawning phase in rivers, and that wild salmonids, mainly trout, are possible carriers of benign wild-type variants of ISAV. Change in virulence is probably a result of deletions of amino acid segments from the highly polymorphic region (HPR) of benign wild-type isolates after transmission to farmed salmon. Hence, it has been suggested that the frequency of new outbreaks of ISA in farmed salmon could partly reflect natural variation in the prevalence of ISAV in wild populations of salmonids. The aims of the present study were to screen for ISAV in wild salmonids during spawning in rivers and to determine the pathogenicity of resultant isolates from wild fish. Tissues from wild salmonids were screened by RT-PCR and real-time PCR. The prevalence of ISAV in wild trout *Salmo trutta* varied from 62 to 100% between tested rivers in 2001. The prevalence dropped in 2002, ranging from 13 to 36% in the same rivers and to only 6% in 2003. All ISAV were non-pathogenic when injected into disease-free Atlantic salmon, but were capable of propagation, as indicated by subsequent viral recovery. However, non-pathogenic ISAV has also been found in farmed salmon, where a prevalence as high as 60% has been registered, but with no mortalities occurring. Based on the results of the present and other studies, it must be concluded that vital information about the importance of wild and man-made reservoirs for the emergence of ISA in salmon farming is still lacking. This information can only be gained by further screening of possible reservoirs, combined with the development of a molecular tool for typing virulence and the geographical origin of the virus isolates.

KEY WORDS: ISA virus · Wild salmonids · Natural reservoir

INTRODUCTION

Infectious salmon anaemia virus (ISAV) is the etiological agent of a disease that has occurred in most salmon *Salmo salar*-producing countries worldwide (Thorud & Djupvik 1988, Mullins et al. 1998, Rodger et al. 1998, Rowley et al. 1999, Bouchard et al. 2001, Kibenge et al. 2001, Ritchie et al. 2001). The official history of ISA dates back to 1984 in Norway, but the virus must be much older, being a distant relative of other viruses in the family Orthomyxoviridae (Krossøy et al. 1999). Based on the mutation rate of the RNA-dependent RNA polymerase (ISAV Segment 2) and hemagglutinin genes (Segment 6), North American and European isolates are thought to have separated >100 yr ago (Krossøy et al. 2001, Krossøy 2002, Nylund et al. 2003). The demonstrated existence of ISAV on both the European and North American continents

prior to the advent of intensive salmon aquaculture, suggests the long-term maintenance of ISAV in a wild reservoir. ISAV and raised levels of ISAV-specific antibodies have been found in wild salmonids with no clear relationship to aquaculture activity (Raynard et al. 2001a, K. Falk pers. comm.). Furthermore, studies based on sequencing of a so-called highly polymorphic region (HPR) of the haemagglutinin gene have suggested that all pathogenic ISAV isolates may be derived by differential deletions or by recombination of a potential ancestral sequence obtained from wild fish (Devold et al. 2001, Cunningham et al. 2002, Mjaaland et al. 2002, Nylund et al. 2003). Coupled to the fact that a range of salmonids have been experimentally shown to propagate ISA (Nylund et al. 1994a, 1995a,c, 1997, Nylund & Jakobsen 1995, Rolland & Nylund 1998a, Devold et al. 2000, Snow et al. 2001a,b, Rolland & Winton 2003), this evidence suggests that wild salmonids may constitute natural host species within which an avirulent ISAV subtype may have co-evolved.

The most frequent interactions between individual wild salmonids and, hence, opportunities for ISAV transmission between hosts occur during spawning in rivers. The rivers are also the rearing areas for susceptible fry, parr and smolts, i.e. individuals that can be expected to lack the acquired immunity towards viral diseases. Based on these facts it is expected that a virus, which has co-evolved with salmon/trout, should be adapted to transmission in the freshwater phase, i.e. in the rivers and preferably during spawning when the chances of interactions between individuals are highest. It has been documented that the production of ISAV in trout increases during stress and sexual maturation (Nylund et al.1994a, 1995a, Rolland & Nylund 1998a, Devold et al. 2000). It has also been thoroughly documented that the ISAV can be transmitted in both fresh- and seawater, and it has been shown that the virus can be present in the mucus, urine, faeces and ovarian fluids of infected individuals (Thorud & Djupvik 1988, Thorud 1991, Nylund et al. 1993, 1994a,b, 1995a,b, 1997, 1999, Hovland et al. 1994, Nylund & Jakobsen 1995, Totland et al. 1996, Rolland & Nylund 1998a,b, Jones et al. 1999, Melville & Griffiths 1999, Devold et al. 2000, Jones & Groman 2001, Raynard et al. 2001b, Snow et al. 2001a,b).

Wild trout and salmon from 5 different rivers were collected and screened for the presence of ISAV. Two different

areas in western Norway with frequent outbreaks of ISA in farmed salmon were selected, and fish were collected during spawning in the rivers in late October of 2001, 2002 and 2003. Pathogenicity characteristics of resultant ISAV were tested by intraperitoneal injection into disease-free salmon.

MATERIALS AND METHODS

Sample collection. Wild salmonids, trout *Salmo trutta* and salmon *Salmo salar*, from 5 selected rivers (Austgulen, Nordgulen, Takle, Brekke and Bortne) in western Norway were collected on 23 October 2001, on 29/30 October 2002 and on 28/29 October 2003 (Fig. 1). All 5 rivers are located in Sogn- og Fjordane county, in areas that traditionally have had a high density of salmon farms. Bortne River is located in the outer part of Nordfjord, while the other 4 rivers are located in the outer part of Sognefjord. Both areas have experienced frequent outbreaks of ISA since the early 1990s. Fish from these rivers were obtained by electrofishing from the river mouth and a maximum of 1 km up the rivers (Table 1). In the spring of 1998, 45 sea trout *S. trutta* were collected by trawl from the outer part of Nordfjord, i.e. the same fjord where the Bortne River is located.

Tissue samples from gills, heart, liver, kidney, pseudobranch, gut, spleen and gonads were taken from the newly killed fish and immediately frozen in
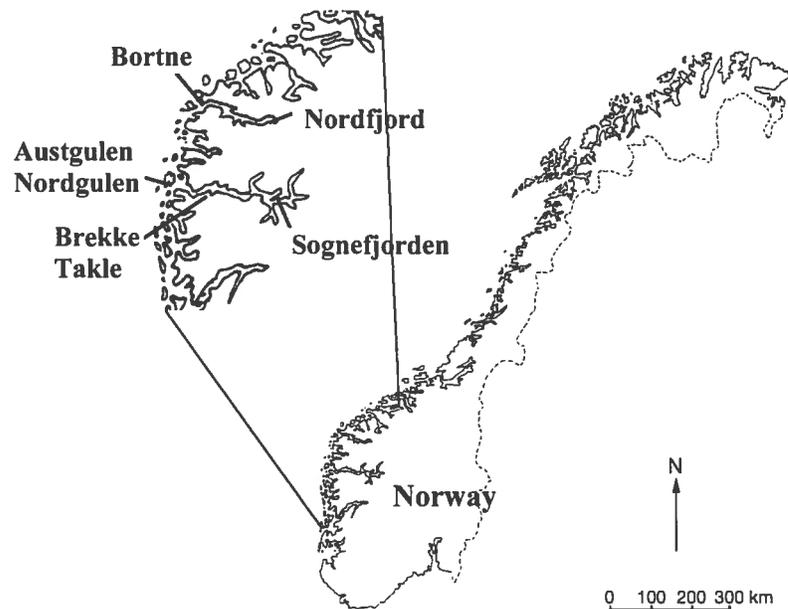

Fig. 1. Location of the rivers in western Norway where the trout and salmon were collected in 2001, 2002 and 2003

Table 1. *Salmo trutta, S. salar, Oncorhynchus mykiss*. Material collected from 5 rivers in 2001, 2002 and 2003 and 1 fjord in 1998 (L-range: size range [cm] of the collected fish)

	Species	N	Male	Female	Not mature	L-range	Year
Rivers							
Nordgulen	S. trutta	21	10	10	1	18.5–47.0	2001
	S. trutta	24	14	6	4	18.5–50.0	2002
	S. trutta	25	15	10	11	10.0–41.5	2003
Brekke	S. trutta	12	5	5	2	17.5–47.5	2001
	S. salar	4	–	4	–	50.0–60.0	2002
	S. trutta	26	8	8	10	19.0–50.0	2002
	S. trutta	27	20	7	7	13.5–65.0	2003
Austgulen	S. trutta	13	3	4	6	17.5–35.0	2001
	O. mykiss	1	1			22.5	2001
Takle	S. trutta	4	2	2	–	16.5–21.0	2001
Bortne	S. salar	3	3	–	–	60.0–80.0	2002
	S. trutta	24	10	4	10	20.0–60.0	2002
	S. salar	1	–	1	1	10.5	2003
	S. trutta	16	10	6	7	10.5–31.0	2003
Fjord							
Nordfjord	S. trutta	45	–	–	45	–	1998

liquid nitrogen. On arrival at the laboratory, the tissues were transferred to –80°C.

Virus isolation and detection. Culture of ISAV was attempted by inoculation of positive tissues on Atlantic salmon kidney (ASK) cells (Devold et al. 2000) using 3 passages. Positive cell cultures, i.e. cell cultures showing a cytopathic effect (CPE), and negative cell cultures were all tested for possible presence of ISAV by RT-PCR (primers: FA-3/RA-3) (Devold et al. 2000).

ISAV-positive homogenates of tissues from salmon in each river (with the exception of the Takle River) were injected intraperitoneally into 30 disease-free salmon, to see if any mortality would occur and if the virus could be cultured in salmon. The tissue samples from wild and experimentally challenged fish were screened by RT-PCR using the diagnostic primers FA-3/RA-3 (Devold et al. 2000) and by real-time PCR. Extraction of RNA from the tissues, reverse transcription polymerase chain reaction (RT-PCR) and sequencing of PCR products were performed as described by Devold et al. (2000). The results of the RT-PCR and real-time PCR assays on the wild fish were tested by contingency table analysis to see if prevalence was significantly different in the 3 following years.

The reaction mix for the real-time PCR was as follows: 1 µl of single-stranded cDNA from the reverse tran-scription, 12.5 µl TaqMan Universal Master Mix (2×), 900 nM of each primer and 200 nM of the MGB probe. The total volume was adjusted to 25 µl using RNAse-free water. The reaction cycle was 2 min at 50°C (UNG activation), 10 min at 95°C and 45 cycles of 95°C for 15 s, and 1 min at 60°C. Two different real-time assays were designed for detecting the ISAV genome: 1 assay for Segment 7 (the tentative nonstructural protein genes) and 1 assay for Segment 8 (the tentative matrix protein genes) (Table 2). An 84 bp and a 63 bp region were amplified from Segments 7 and 8, respectively. The labelled fluorogenic probes are specific for the cDNA region between the forward and reverse primer sets. The probes contain a fluorescent reporter dye and a quencher dye; their physical proximity to each other suppresses any light emitted by the reporter. During PCR, the 5' → 3' nuclease activity of the DNA polymerase releases the reporter dye from the probe, which results in an increase in fluorescence. The reporter dye, 6-FAM, has an emission wavelength maximum of $\lambda_{max} = 518$ nm and is measured by the ABI 7700 sequence detection system (Applied Biosystems).

The sensitivity of the real-time PCR assays was compared with the sensitivity of the diagnostic PCR using the primer set FA-3/RA-3 (Devold et al. 2000). A dilution series of cDNA from ISAV was used as templates in the comparison of the different assays.

In 2003 the tissue distribution of virus in ISAV-positive fish was tested by real-time PCR. Originally, only kidney tissue was tested for the presence of virus, as was done during 2001 and 2002 as well.

Table 2. Primer and probes in the assay mix were produced by Applied Biosystems (ABI). The probes are specific for the region between the primer sets and are targeted against Segments 7 and 8 from the ISAV (infectious salmon anaemia virus) genome

Primers/probes	Name	Sequence
Segment 7		
Probe	S7-P1	6FAM-cac atg acc cct cgt c-MGBNFQ
Forward primer	S7-F1	5'-tgg gat cat gtg ttt cct gct a
Reverse primer	S7-R1	5'-gaa aat cca tgt tct cag atg caa
Segment 8		
Probe	S8-P1	6FAM-acg gtg gat ctt tc-MGBNFQ
Forward primer	S8-F1	5'-cga cga tga ctc tct act gtg tga t
Reverse primer	S8-R1	5'-tca tca gtg tcg cca tgc tt

Sequencing and sequence analysis. PCR products were purified using Qia-quick PCR purification columns (Qiagen) and then sequenced using the Big Dye terminator Sequencing kit (Applied Biosystems). The templates were sequenced in both directions. Sequence data were assembled with the help of Vector NTI software (InforMax), and GenBank searches were performed using BLAST (2.0).

The Vector NTI Suite software package (InforMax) was used for the multiple alignments of partial nucleotides. To perform pairwise comparisons between the different sequences from the ISAV isolates, the multiple sequence alignment editor GeneDoc was used. Phylogenetic analyses of the data sets were performed using PAUP* Version 4.0 (Swofford 1998) and TREE-PUZZLE 5.0 (available at: www.tree-puzzle.de). TREE-PUZZLE reconstructs phylogenetic trees from molecular data by maximum likelihood, and computes maximum-likelihood distances and branch lengths. Phylogenetic trees were drawn using TreeView (Page 1996).

RESULTS

Trout *Salmo trutta* were the dominating salmonid species in the rivers and fjords where the fish were collected and screened for the presence of ISAV. The sample material also included a few Atlantic salmon *Salmo salar* from 2 rivers (Bortne & Brekke) and 1 individual of rainbow trout *Oncorhynchus mykiss* collected in the Austgulen River. The majority of fish from the rivers were spawning or had just spawned, but a few parr and smolt were also included in the collected material. Except for the rainbow trout, none of the fish seemed to be escapees from fish farms. The weight of the fish was not measured, but their length, the number of fish caught, their sex and the number of immature fish are given in Table 1. The mean length of the fish caught in 2003 was much smaller and more fish were immature compared to those caught during the previous years.

None of the fish showed any clinical signs of ISA. However, 1 spawning salmon from the Bortne River (2002) had skin ulcers, and 1 immature trout from the Brekke River (2003) had white granulomas and an enlarged kidney. In 2003 5 trout (4 immature, 1 spawning) had nodules/vesicles on their spleens, while 1 spawning trout from the Brekke River was infected with *Anisakis simplex*, and 1 spawning trout from the Bortne River was heavily infected with *Eubothrium* sp. All the fish were screened for the presence of ectoparasites. A few of the fish had salmon lice, *Lepeophtheirus salmonis*, *Trichodina* spp. and *Riboscyphidia* sp., but the intensity was low, and no lesions due to parasites were observed.

Virus detection

It was not possible to culture any ISAV in ASK cells from any of the fish included in this study. However, it was possible to detect ISAV in salmon, challenged with tissue homogenates from ISAV-positive fish, 4 wk after challenge, but no mortality was observed. Sequencing of the PCR products and phylogenetic analysis of the sequences, confirmed them to be ISAV genomes of European origin.

The kidneys from all the collected fish were screened for the presence of ISAV with the help of RT-PCR (primers: FA-3/RA-3), and fish collected in 2002 and 2003 were also screened by real-time PCR using primers and probes targeting Segment 8. Both the Segment-7 and -8 real-time PCR assays were used in the screening of the material from 2003. The real-time PCR assays were more analytically sensitive than the RT-PCR (FA3/RA3), and gave positive reactions with 1000 times less template than the RT-PCR (Fig. 2). The results of the RT-PCR and real-time tests are presented in Table 3, and this table shows a clear trend: the highest prevalence of ISAV was detected in 2001 (up to 100%), it decreased during 2002 to a maximum of 81% and reached a low point in 2003, with no more than 1 fish testing positive for ISAV in each river. This trend can be observed in all rivers and is confirmed by both RT-PCR and real-time PCR. The results of the RT-PCR and real-time PCR assays on the wild fish were tested by contingency table analysis (Table 3). Only 3 of the 45 sea trout collected in 1998 were positive for ISAV.

Unfortunately, only 3 fish were available for testing the tissue distribution of virus in ISAV-positive fish in 2003, 2 that were immature and 1 that was spawning. The results are presented in Table 4 and show that ISAV was only detected in 4 of the 8 tissues tested.

DISCUSSION

Molecular methods have become important tools in viral diagnostics and in the study of the diversity, maintenance and dissemination of viral diseases (Hungnes et al. 2000). The latest revolution in viral diagnostics came with the development of real-time PCR due to its rapidity, sensitivity (i.e. low detection limit), reproducibility and the reduced risk of carry-over contamination (Mackay et al. 2002). In addition, it provides the possibility for relative and absolute quantification. The high sensitivity of real-time PCR makes it a good method for detection of carriers of viral agents like the ISAV, since it must be expected that orthomyxoviruses have to produce a low number of infective particles in order to persist in the host (Knipe & Howley 2001). PCR has established itself during recent years as the gold
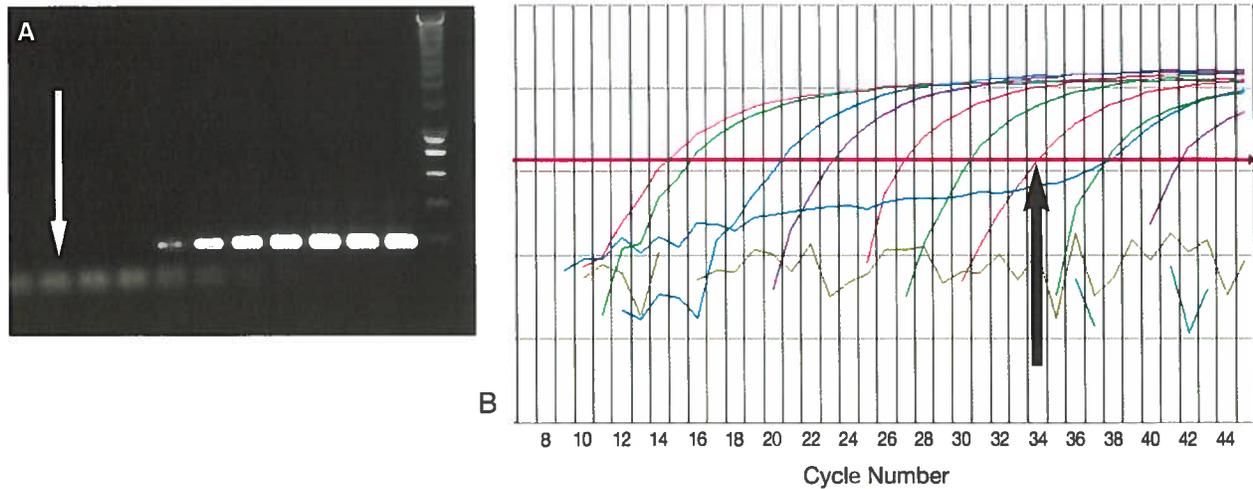
Fig. 2. (A) Demonstrating the difference in threshold of detection of infectious salmon anaemia virus by RT-PCR (FA3/RA3) and the 2 real-time PCR assays targeting Segments 7 and 8. Agarose gel showing RT-PCR amplification products (10 µl) stained by ethidium bromide. The lanes represent 10-fold dilutions. The white arrow marks the dilution that could be detected by the real-time PCR assays. (B) Results of the real-time PCR assay targeting Segment 7 using the same dilution series as in the RT-PCR. The black arrow shows the detection limit for the RT-PCR

standard for detecting nucleic acids from a number of origins, and RT-PCR has become an important tool in the diagnostics of ISA in both Europe and North America (Mjaaland et al. 1997, Devold et al. 2000, Griffiths & Melville 2000, Mikalsen et al. 2001, Raynard et al. 2001a,b, Snow et al. 2001a, 2003, Løvdal & Enger 2002, McClure et al. 2004). The dominating assays for RT-PCR detection of ISAV have been an assay published by Mjaaland et al. (1997) and another assay developed by Devold et al. (2000). Both assays target Segment 8, presumably the matrix protein genes from the ISAV (Biering et al. 2002). The real-time PCR used in the present paper is able to detect template 1000 times more dilute than the RT-PCR assay (FA3/RA3) developed by Devold et al. (2000). This difference in sensitivity is also reflected by the results presented in this study, in which the real-time PCR in each case gives a higher number of ISAV-positive individuals.

The official history of the ISAV in salmon farming goes back to 1984 in western Norway (Thorud & Djupvik 1988). However, based on the mutation rate of the RNA-dependent RNA polymerase gene, Krossøy et al. (2001) estimated that the European and North American isolates of ISAV must have separated at least 100 yr ago. More than 100 yr of separation between European and North American ISAV isolates was also supported by a study of the mutation rate of the haemagglutinin gene (Segment 6) of the ISAV (Nylund et al. 2003). A dis-

Table 3. *Salmo trutta, S. salar, Oncorhynchus mykiss.* Results of the RT-PCR and real-time PCR screening of kidney tissues from wild trout *S. trutta* and salmon *S. salar* caught in rivers located in western Norway (nt: not tested; n: number of positive fish; %: percentage of positive fish). Results of the RT-PCR and real-time PCR assays on the samples of *S. trutta* collected in 2001, 2002 and 2003 from the Nordgulen, Brekke and Bortne Rivers were tested by contingency table analysis (χ^2/p<). There was a significant decline of ISAV-positive trout in all 3 rivers using both assays for detection

	Species	N	RT-PCR n/%	χ^2/p<	Real-time PCR n/%	χ^2/p<	Year
Rivers							
Nordgulen	*S. trutta*	21	14/67		nt		2001
	S. trutta	24	3/13	30.3/0.001	9/38		2002
	S. trutta	25	0/0		1/(4)	8.5/0.01	2003
Brekke	*S. trutta*	12	8/67		nt		2001
	S. salar	4	3/75		4/(100)		2002
	S. trutta	26	7/27	21.1/0.001	21/81	32.4/0.001	2002
	S. trutta	27	0/0		1/(4)		2003
Austgulen	*S. trutta*	13	13/100		nt		2001
	O. mykiss	1	1/100		nt		2001
Takle	*S. trutta*	4	3/75		nt		2001
Bortne	*S. salar*	3	0/0		2/(67)		2002
	S. trutta	24	8/34		15/63		2002
	S. trutta	16	0/0	6.6/0.01	1/(6)	12.7/0.001	2003
	S. salar	1	0/0		0/(0)		2003
Fjord							
Nordfjord	*S. trutta*	45	3/6.7		nt		1998

Table 4. *Salmo trutta, S. salar, Oncorhynchus mykiss*. Tissue distribution of ISAV in positive carrier fish from 3 rivers (Bortne, Brekke, Nordgulen) using real-time PCR. The first PCR was done on kidney tissue only to check for absence/presence of ISAV; the second PCR was done on various tissues from ISAV-positive fish in order to determine tissue distribution of virus. Superscript 7 and 8: PCR for Segment 7 and 8, respectively. nt: not tested; Ct: cycle thresholds of the growth curves

	Tissue	Ct		
		Bortne	Brekke	Nordgulen
1st PCR	Kidney	36.96[7]	38.12[7]	39.29[8]
2nd PCR	Kidney	30.88[7]/29.16[8]	–	–
	Pseudobranch	–	–	–
	Gill	–	–	–
	Heart	–	36.95[8]	38.08[8]
	Liver	–	–	–
	Spleen	32.51[7]	–	–
	Gonads	nt	nt	–
	Gut	40.22[8]	–	–

tant relationship to the other members in the family Orthomyxoviridae, the influenza viruses (Krossøy et al. 1999), suggests a very old and probably a marine origin for the ISAV. This has led to a hunt for the marine reservoirs of ISAV, and many marine fish species have been challenged to see if they could be hosts for the ISAV (Thorud & Torgersen 1994, Kvenseth 1998, Nylund et al. 2002, Snow et al. 2002). So far, only limited replication has been found in herring *Clupea harengus* (Nylund et al. 2002). On the other hand, the ISAV is able to replicate in most salmonid species, *Salmo* spp., *Oncorhynchus* spp. and *Salvelinus alpinus* (Nylund et al. 1994a, 1995a,b, 1997, Nylund & Jakobsen 1995, Rolland & Nylund 1998a, Devold et al. 2000, Snow et al. 2001a,b, Rolland & Winton 2003), causing mortality in Atlantic salmon only (Thorud & Djupvik 1988, Mullins et al. 1998, Rodger et al. 1998, Bouchard et al. 2001). Based on empirical and experimental evidence, everything points towards salmonids as the natural hosts of ISAV, and, in Norway, this means in most cases Atlantic salmon *S. salar* and trout *S. trutta*.

All salmonids have a freshwater phase when they are young, and this early phase is, for most salmonid species, followed by a marine phase (growth phase). At the start of sexual maturation the salmonids at sea return to freshwater, usually rivers, to spawn. Hence, the highest population densities of hosts will be found in rivers during spawning, where, in addition to mature salmonids, there will be an abundance of younger, susceptible stages of the same species (cf. Nylund et al. 2003). In a natural system, the most obvious place to look for the ISAV would, therefore, be in salmonid hosts during spawning. The present study, based on the material collected in 2001 and 2002,

strongly supports such a view. Both RT-PCR and real-time PCR resulted in a high number of positive individuals of trout in both years, but higher in 2001 compared to 2002. However, the number of positive individuals dropped significantly in 2003 when only 1 individual fish was found positive in each of the 3 rivers that were screened. In 1998 a similar screening for ISAV was performed (RT-PCR using FA3/RA3) on sea trout (N = 45) collected in Nordfjord (the fjord where the Bortne River empties) and 3 individuals, 6.7%, were found to be positive. Salmonids in freshwater in the UK have also been found positive for ISAV, and it was suggested that they were infected by transmission of virus from ISAV-positive marine farming sites (Raynard et al. 2001a). Hence, important questions are: What connections exist between wild and farmed salmonids? Can changes in infection pressure from ISAV-positive salmon farms explain the large drop in number of ISAV-positive wild fish from 2001 to 2003? Does the pattern of ISAV prevalence in wild fish reflect the infection pressure from marine salmon farms or vice versa, or is the observed pattern just reflecting natural variation within these populations?

Most marine salmon farms close to these rivers were empty in the period summer 2002 to summer 2003 due to earlier problems with ISA. They have now been restocked. On the other hand, it is also known that the prevalence of virus in natural populations may vary from year to year depending on the status of acquired immunity within the respective populations (Knipe & Howley 2001). Testing of the ISAV from the wild fish showed that none of these gave any pathology or mortality when injected into disease-free salmon, but they did replicate, i.e. the ISAV were still detected 4 wk after the challenge. Avirulent ISAV have also been detected in farmed salmon in western Norway (A. Nylund unpubl. data), and, hence, farmed salmon could have been responsible for the high prevalence of ISAV in the rivers in 2001 and 2002. However, this would not be the pathogenic ISAV isolates collected from farms in these 2 areas in the period from 2000 to 2003. To better understand the relationship between the prevalence of ISAV in wild salmonids and the occurrence of ISA in farmed salmon, it will be necessary to screen wild populations of salmonids over a longer period (maybe 10 yr). The results from such a study should be correlated with data on ISA from salmon farms in the same areas. In addition, if possible, a molecular typing system for geographical origin and virulence should be developed to aid the interpretation of the results from such a study.

It is obviously too early to have any firm opinions about what is happening in the western Norwegian river systems included in this study. However, the

occurrence of ISAV in Norway, Canada, the UK, the Faeroe Islands, the USA and Ireland represent separate events, including different ISAV isolates (Nylund et al. 2003). In all these cases, the emergence of ISAV must have local natural reservoirs (nothing is known about the Irish ISAV). This assumption is further supported by the discovery of ISAV, in Scotland and Canada, with full-length HPRs in the HA gene (Segment 6), i.e. sequences not previously observed in any other countries (Cunningham et al. 2002, Cook-Versloot et al. 2004). Full-length HPRs were later also found in ISAV from farmed salmonids in the USA and Norway (A. Nylund unpubl. data), and these viruses are different from each other and from those described in the UK and Canada. A study of ISAV isolates from Norway, based on sequences of the HA gene, shows that there are several distinct groups of ISAV in Norway (Devold et al. 2001, Nylund et al. 2003). These isolates reflect geographical origin and time of collection, and, based on this information, Nylund et al. (2003) suggest that at least 21 transmissions from wild to farmed salmonids are represented in the material presented. Still, it is a long way from the estimated number of transitions from wild to farmed fish that can be documented to the hundreds of outbreaks of ISA in salmon farming through the years. A number of these outbreaks can be explained as horizontal transmissions between salmon farms and spreading of ISAV via transportation of infected smolts, but the tools needed to quantify this type of transmission are not available. Hence, there are large numbers of ISA cases in salmon farming for which transmission from wild fish or between salmon farms cannot be determined. Future management of ISA does, however, require knowledge about the source of the ISAV responsible for new outbreaks. We, therefore, recommend that more resources should be put into the study of natural reservoirs, man-made reservoirs (farmed stocks of salmonids) and the development of better molecular tools for typing of ISAV isolates with respect to geographical origin and virulence.

The work on tissue distribution, presented in this study, is limited to 3 individuals only (*Salmo trutta*). Two of these specimens were young fish (parr), and the gonads were not collected from these. The results from the tissues screened provides no clear pattern that can be used to support or reject any hypothesis about tissue/cell tropism in carriers, nor can the results be used to test any hypothesis about interspecies transmission of the ISAV. Cycle threshold (Ct) values from 38 and up are close to the detection limit for this real-time PCR and are not always reproducible. This explains why the second PCR on the kidney samples from Brekke and Nordgulen was negative. Further studies on tissue distribution of ISAV in carriers are needed.

The present study has shown that trout and salmon in Norwegian rivers are carrying ISAV and that these species could be the natural reservoirs for this virus. The importance of this natural reservoir, with respect to ISA in farmed salmon, has not been determined, nor can any conclusions be made about the influence of ISAV-infected farmed salmon on wild populations and their carrier status. These important questions for the management of ISA will require comparative studies of natural and man-made reservoirs over a longer timescale. Such studies will also rely upon the development of a typing system for the ISAV with respect to geographical origin and virulence.

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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

Debes H. Christiansen,[1] Peter S. Østergaard,[1] Michael Snow,[2] Ole Bendik Dale[3] and Knut Falk[3]

Correspondence
Debes H. Christiansen
Debesc@hfs.fo

[1]Food and Veterinary Agency, National Reference Laboratory for Fish Diseases, Tórshavn, Faroe Islands

[2]Marine Scotland Science, Marine Laboratory, Aberdeen, Scotland, UK

[3]National Veterinary Institute, Section for Fish Health, Oslo, Norway

Infectious salmon anemia virus (ISAV) is an orthomyxovirus responsible for a significant disease of farmed Atlantic salmon. Fallowing 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 & Djupvik, 1988), the disease spread rapidly throughout the country resulting in a peak of more than 80 outbreaks per year in 1990 (Håstein *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

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.

Year	Kidneys			Gills		
	Total	ISAV positive		Total	ISAV positive	
	n	*n*	%	*n*	*n*	%
2005	2 998	0	0	–	–	–
2006	7 157	10	0.1	–	–	–
2007	6 505*	142	2.2	5 387*	811	15.1
2008	–	–	–	9 066	1 100	12.1
2009	–	–	–	8 847	852	9.6
Total	16 660	152	0.9	23 300	2 763	11.9

*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 ISAV

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 throughout the study period, suggesting that the presence of ISAV-HPR0 does not result in classical ISA disease or other signs of disease. 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

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

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.

|		Kidneys			
		Negative		Positive	Total
Gills	Negative	480		14 **35.9** [31.4–36.5]	494
	Positive	474 **31.4** [18.6–37.9]		127 Gills=**28.1** [19.0–36.7] Kidneys=**35.7** [27.1–38.3]	601
	Total	954		141	1095
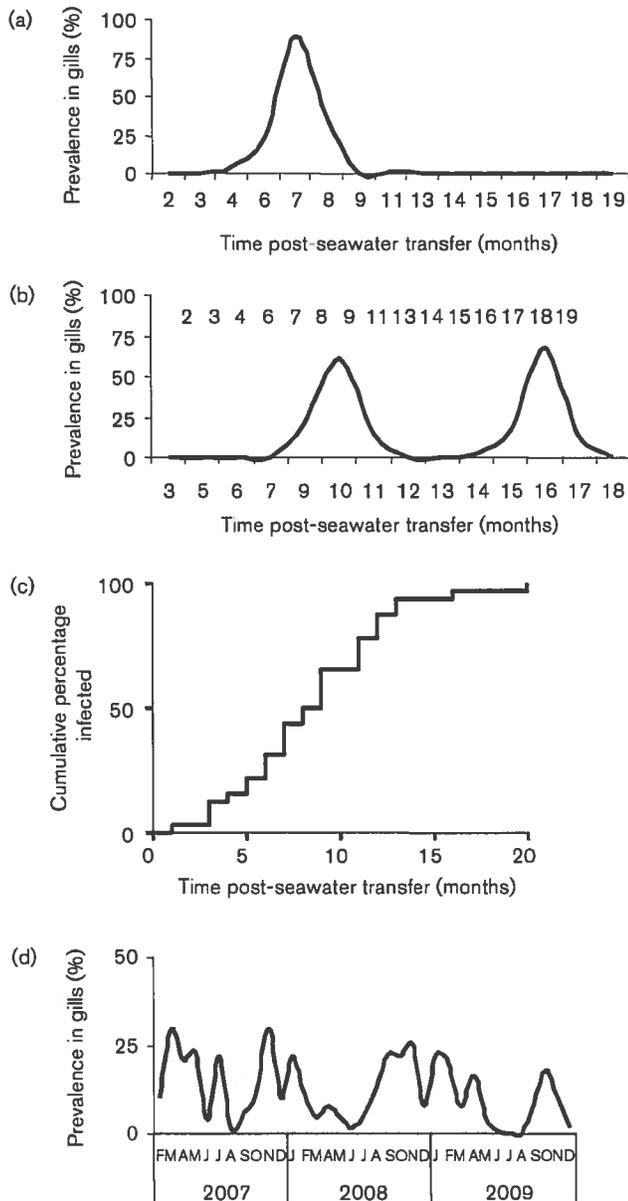
Fig. 1. ISAV-HPR0 infection pattern in Faroese Atlantic salmon marine grow-out sites. Examples of two infection patterns throughout the production cycle are outlined in (a) and (b). (a) Example of a farm with only one ISAV-HPR0 infection peak, at 7 months post-seawater transfer. (b) Example of another site with two infection peaks, the first peak 10 months and the second peak 16 months post-seawater transfer. (c) Outline of the time from post-seawater transfer to initial detection of ISAV-HPR0 shown as cumulative percentage infected for all 36 ISAV-HPR0-positive production cycles. The mean time of infection was 8.5 months post-seawater transfer (range 1–20 months). (d) Overall monthly prevalence of ISAV-HPR0 infection at all sea sites producing Atlantic salmon from 2007 to 2009. Prevalence appeared to peak during the winter. The mean number of fish examined per month for ISAV was 626 (range 151–1003).

(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 C_t value of 28 (range 17–36) whereas samplings with a mean prevalence <20 % gave a mean C_t 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.
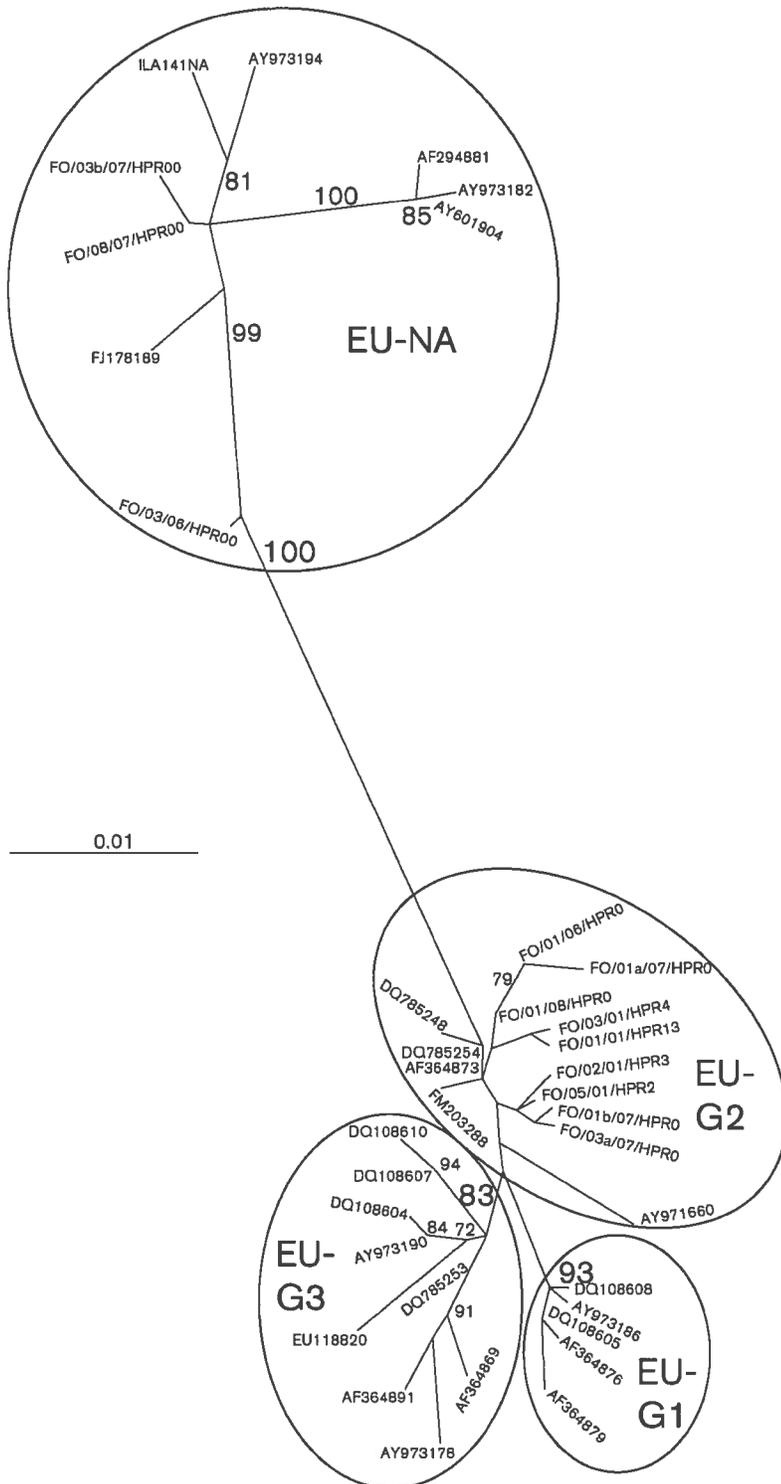
Fig. 2. Phylogenetic radial tree showing the relationships between 12 Faroese ISAV sequences and 25 representative ISAV sequences from each of the four established major European-like subgroups (EU-G1–G3) circulating in Europe and the European-like subgroup circulating in North American (EU-NA) previously 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/01/08/HPR0, HQ664999. Bar, 0.01 substitutions per nucleotide side.

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

proven sensitivity of real-time PCR with an intensive and optimized sampling method focussing 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, C_t 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 C_t 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 seawater transfer might suggest introduction from an 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 fallowing 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 $\pm$ 10 mg) from each fish were placed in 500 µl RLT-lysis 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 RNeasy 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'-GGCTATCTACCATGAACG-AATC-3') and ISAs8-1R (5'-CCGCCAAGTGTAAGTAGCACTC-3') (Mjaaland *et al.*, 1997). In addition, the reaction mixture included the following primers MHC IIβ-1F: 5'-TCAGATTCAACAGCACTG-TGGG-3' and MHC IIβ-1R: 5'-TACTACAGCACCCCAGAAGAC-3' (DNAtechnology) for amplification of a 577 bp specific fragment of the Atlantic salmon *MHC* IIβ gene (GenBank accession no. X70167) as an internal control (IC). The reaction mixture was as 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

(Qiagen). An ISAV segment 8-specific fragment was amplified with the following primers 5'-CGAAAGCCCTGGAAACTTTAGA-3' and 5'-GATGCCGGAAGTCGATGAACT-3', and a MGB TaqMan probe (FAM-aaggccatcgtcgct-NFQ; Applied Biosystems). A specific fragment of the Atlantic salmon housekeeping gene $EF1\alpha$ (GenBank accession. no. AF321836), as IC, was amplified with the primers 5'-GGCTGGTTCAAGGGATGGA-3' and 5'-CAGAGTCACACCATTGG-CGTTA-3', and a MGB TaqMan probe (VIC-tcgagcgtaaggatg-NFQ).

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/elongation at 60 °C for 60 s. Amplicons were analysed at thresholds of 0.001 ($EF1\alpha$) and 0.005 (ISA segment 8). The mean C_t value of the $EF1\alpha$ of the 17 913 gill samples analysed in 2008–2009 was 16.3 (SD=0.87). Thus, the ISAV real-time RT-PCR demonstrated a highly reproducible performance.

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), following the protocol of the manufacturer, and analysed on an ABI 3100 Avant (Applied Biosystems).

Primers and TaqMan probes. Primers and probes were designed with Primer Express 3.0 (Applied Biosystems) and FastPCR (Primer digital.com). All primer/probes met the relevant design requirements including T_m, hairpin, homodimer, heterodimer and specificity. In addition primers and probes were evaluated using the nucleotide BLAST search in GenBank.

Controls. Controls were implemented in each PCR run to monitor the performance of the RT-PCR and real-time RT-PCR assays. To monitor the purity of the RNeasy 96 reagents, reagent controls were processed on each RNeasy 96 silica plate without organ material added. To monitor the purity of the RT-PCR and real-time RT-PCR reagents, non-template controls without RNA template were conducted. To monitor the PCR efficiency of the assay a standard curve using a tenfold dilution series of ISAV-positive RNA was employed (Snow et al., 2006). To monitor sample quality and to control for pipetting errors simultaneously as ISAV was detected, a normal cellular gene sequence was used as an IC in the doubleplex RT-PCR and real-time RT-PCR assays (Mackay et al. 2002). If IC bands in the RT-PCR showed clearly weaker intensity than the normal bands by visual inspection, the sample was excluded. If the IC of a sample showed a C_t value more than one log greater by real-time RT-PCR than the mean C_t value of the IC in the positive controls, the sample was excluded.

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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Development, Application and Validation of a Taqman® Real-Time RT-PCR Assay for the Detection of Infectious Salmon Anaemia Virus (ISAV) in Atlantic Salmon (*Salmo salar*)

M. Snow[1], P. McKay[1], A.J.A. McBeath[1], J. Black[1], F. Doig[1], R. Kerr[1], C.O. Cunningham[1], A. Nylund[2], M. Devold[2]

[1] FRS Marine Laboratory, Aberdeen, U.K.
[2] Department of Biology, University of Bergen, Bergen, Norway

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Abstract: Infectious salmon anaemia (ISA) is a disease of cultured Atlantic salmon (*Salmo salar*) which was successfully eradicated from Scotland following its emergence in 1998. The rapid deployment of sensitive diagnostic methods for the detection of ISA virus (ISAV) was fundamental to the swift eradication of ISA disease in Scotland and continues to be of crucial importance to surveillance of the aquaculture industry. This study reports the development, validation, application and interpretation of two independent, highly sensitive and specific semi-quantitative Taqman* real-time RT-PCR (qRT-PCR) methods for the detection of ISAV. Such technology offers considerable advantages over conventional RT-PCR methods in current routine use for ISAV surveillance. These include an increased sensitivity, enhanced specificity, semi-quantification using endogenous controls, a lack of subjectivity in results interpretation, speed of processing and improved contamination control.

INTRODUCTION

Infectious salmon anaemia virus (ISAV) is a pathogen of cultured Atlantic salmon (*Salmo salar*) which has caused significant production losses in Norway, Scotland, Canada, the U.S.A., and the Faroes. Emergence of ISA disease in Scotland in the late 1990s prompted a swift and apparently successful eradication programme [1]. The disease has, however, become established in Norway and Canada where in 1999 alone it was estimated to be responsible for losses totalling $11 and $14 million, respectively [2].

The rapid deployment of sensitive diagnostic methods for the detection of ISAV

was fundamental to the swift eradication of ISA disease in Scotland and remains equally important in managing the impact and spread of this virus in counties where it is endemic. Three diagnostic assays are in widespread use for ISAV diagnosis, which include virus isolation on permissive cell lines such as SHK-1 [3], indirect fluorescent antibody testing (IFAT) [4] and reverse transcriptase polymerase chain reaction (RT-PCR) [5]. Of these assays, the RT-PCR is generally regarded as the most sensitive [6-8] although a recent study highlighted a lack of reproducibility between laboratories and emphasized a need to standardize inter-laboratory protocols [9]. Much of the variability was attributed to the use of different external controls and methods applied to interpret and confirm results [9].

Recent developments in real-time PCR technology offer the potential to overcome many of the limitations of conventional PCR including an improved sensitivity of ISAV diagnostics. Indeed, a sensitivity increase of 100-fold over conventional RT-PCR was recently reported using a SYBR green-based assay for the detection of ISAV [10]. Further advantages of Taqman® real-time PCR chemistry include improved specificity of amplification, since signal generation is reliant on the annealing of a highly specific secondary probe to the primary amplification product. In addition the development of an endogenous control allows the confirmation of target integrity as well as facilitation of relative quantification of viral load and subsequent comparison between samples. Real-time PCR thus offers considerable advantages over conventional RT-PCR-based protocols and has the potential to reduce problems in the interpretation and reproducibility of results between laboratories.

In this report we describe the development, validation and application of Taqman®-based assays targeting two independent genes of ISAV coupled to an assay targeting the constitutively expressed endogenous control translation elongation factor 1 alpha (ELF1α) [11]. The comparative efficiencies of these assays were determined and a method for the relative quantification of viral target-level developed. Assays were compared with the conventional RT-PCR in widespread use for ISAV diagnostics [5] throughout an experimentally induced clinical outbreak of ISAV and in samples analysed for routine ISAV diagnostics in our laboratory in 2004.

MATERIALS AND METHODS

Development of Taqman Real-Time RT-PCR method

Primers and probe design

RT-PCR primer pairs and Taqman® MGB probes targeting ISAV segments 7 and 8 were selected from alignments of all available sequences using published sequence data and the Primer Express software (Applied Biosystems), and are detailed in Table 1. Both primers and probes were targeted to conserved gene regions to ensure detection of all documented variants of ISAV. Primers designed for amplification of the salmon ELF1α gene were those recently published by Moore et al. (2005). The ELF probe was designed to target mRNA specifically by spanning two exons such that it would not amplify contaminating salmon genomic DNA.

Organism	Gene target	Upstream primer	Downstream primer	Taqman® trobe	Amplicon size (bp)
ISAV	Segment 7	CAGGGTTGTA TCCATGGTTG AAATG	GTCCAGCCCT AAGCTCAACT C	FAM- CTCTCTCATTG TGATCCC-MGB	155
ISAV	Segment 8	CTACACAGCA GGATGCAGAT GT	CAGGATGCCG GAAGTCGAT	FAM- CATCGTCGCT GCAGTTC-MGB	104
Salmo salar	ELFIα mRNA	CCCCTCCAGG ACGTTTACAA A	CACACGGCCC ACAGGTACA	FAM- ATCGGT#GGTA TTGGAAC	57

RNA extraction

Tissues were stored in 1 ml RNALater (Ambion) and stored at -20°C before RNA extraction. For field samples, 50-100 mg tissue was homogenised in 1ml TRIZOL using a disposable pestle and RNA subsequently extracted according to the manufacturer's protocol (Invitrogen). RNA was finally resuspended in DEPC-treated H_2O, quantified by measuring the OD at 260 and 280 nm on a Genequant Pro spectrophotometer (Amersham Biosciences) and stored at -80°C. In the case of experimental samples, 10mg tissue was homogenised in 600 μl RLT buffer (Qiagen) for 1min using the TissueLyser system (Qiagen). RNA was subsequently extracted using the RNeasy kit according to the manufacturer's protocol for the extraction of RNA from animal tissues (Qiagen). Total RNA was finally resuspended in 200 μl DEPC-treated H_2O and stored at -80°C. For routine diagnostics, negative extraction controls (designated A control) were conducted by performing a blank extraction. Controls were taken through subsequent RT and PCR steps.

Reverse transcription

For field diagnostic samples, reverse transcription reactions were conducted by first preparing dilutions containing 2 μg RNA in 9.5 μl DEPC-treated dH2O. RNA samples were incubated at 55-60°C for 10min and then placed on ice. Reverse transcription was conducted in a total volume of 20 μl which contained 9.5 μl RNA prepared as above, 5 ngμl⁻¹ random hexamers (Promega), 1x 1ˢᵗ strand buffer, 1mM each dNTP, 1Uμl⁻¹ RNase OUT and 10Uμl⁻¹ MMLV reverse transcriptase (all from Invitrogen). Reactions were incubated at 37°C for 1h. In the case of experimental samples, reverse transcription was performed using the Taqman® RT Kit (Applied Biosystems) in a final reaction volume of 25 μl. Firstly, 9.125 μl RNA & 1.25 μl of 50 μM oligo d(T)₁₆ were mixed and denatured at 70°C for 10 min. Reactions were placed on ice and mixed with 1x reverse transcriptase buffer, 5.5 mM MgCl₂, 2 mM dNTPs, 0.4U RNase Inhibitor and 1.25U Multiscribe reverse transcriptase. Reactions were incubated at 48°C for 90 min followed by 95°C for five min. Negative reverse transcription controls were conducted by performing reactions containing no target (designated B control). Controls were taken through subsequent PCR steps.

Real-time RT-PCR (qRT-PCR) reagents and cycling

Assays were performed on an Applied Biosystems 7000 Sequence Detection System programmed to conduct the following cycling profile: 50°C for two min (AmpErase uracil N-glycosylase incubation), 95°C for 10 min (AmpliTaq Gold polymerase activation) followed by 45 cycles of 95°C for 15s (denaturation) and 60°C for one min (annealing/extension). Reactions were conducted in a final volume of 25 μl containing 1 μl cDNA template, 1x Taqman® Universal PCR mastermix, 900nM each primer and 250 nM Taqman® probe. Controls were conducted for each primer and probe set containing no target (designated C control).

Validation of reaction efficiencies and relative quantification

To determine whether the salmon host cell endogenous ELF1a assay control might permit the relative quantification of ISAV and ensure efficient performance of each assay, a validation experiment was conducted to determine whether the efficiency of all assays was comparable. For each assay, triplicate reactions were conducted on each dilution of a 10-fold serial dilution of cDNA prepared from concentrated standards. Standard curves were generated by plotting the relative dilution of RNA versus the cycle number required to elevate the fluorescence signal above the threshold for the ISAV Segment 7, 8 and ELF assays. Reaction conditions with respect to probe and primer concentrations were independently evaluated as described in the Applied Biosystems literature. The effect of reducing the total reaction volume from 50 µl to 25 µl was also determined.

Sensitivity

The same 10-fold dilution series of ISAV cDNA was used to evaluate the comparative sensitivity of both Taqman® qRT-PCR methods with that of conventional RT-PCR. Triplicate individual reactions were conducted for each test using the methods described above.

Specificity

ISAV is the only orthomyxovirus from fish to be characterised at the molecular level, and as such has been designated within the genus Isavirus. Nucleotide sequence of probes was used to search GenBank using BLAST X [12] to identify any known organisms with which they might cross-react. Segment 7 and 8 probes and primer sets were tested against cDNA derived from a range of other RNA viruses capable of infecting Atlantic salmon including Atlantic salmon paramyxovirus, viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and pancreas disease virus (PD). To ensure the detection of all forms of ISAV, segment 7 and 8 primers and probes were designed from sequence alignments containing representatives of all known subtypes of ISAV, which are classified according to the sequence of a highly polymorphic region (HPR) of the haemagglutinin esterase (HE) gene. Such alignments included the HPR0 subtype from which all other HPR subtypes of the virus which are associated with clinical disease in aquaculture may have been derived [13,14].

Application of relative quantification to the detection of ISAV in experimentally infected fish

Pathogen-free fish

Atlantic salmon post smolts (mean weight +/- SE, 89.4 +/- 14.69 g) were reared at the FRS Marine Research Unit, Aultbea, Ross-shire, Scotland. Before the experimental challenge, four fish were screened for the presence of ISAV using RT-PCR and qRT-PCR as described above. Fish were allowed to acclimatize for seven days and starved for 24 h before experimental infection. Water temperatures were maintained at 11°C for the duration of the experimental infection. Seawater was extracted from the North Sea and passed through a sand filter and UV disinfection treatment before entering fish tanks.

Experimental infection with ISAV

Atlantic salmon post smolts (n=75) were anaesthetised by immersion in methane tricaine sulphonate (3-aminobenzoic acid ethyl ester of MS-222; Sigma) at a concentration of 100 mg l-1, infected intra-peritoneally with a dose of 2×10^4 TCID$_{50}$ fish⁻¹ in an inoculum volume of 100 µl, and distributed between two 360 l aquaria (Tanks 1 & 2). Controls consisted of 75 fish similarly anaesthetised and injected with an equivalent volume of culture medium containing no virus, and distributed between a further two tanks (Tanks 3 and 4). Two additional tanks were similarly set up, each of which contained 25 virus infected and non-infected fish respectively. These tanks were used to monitor the efficacy of the viral challenge. All fish were monitored for the duration of the experimental infection period (20d) and all dead fish were recorded and removed twice daily.

Sampling and comparative PCR analysis

From each set of virus-infected (Tanks 1 and 2) and control tanks (Tanks 3 and 4), a total of four fish were sacrificed and sampled on each of days 1,2,3,4,5,6,7,9,11,15 and 19 post infection. Kidney tissue was excised, sampled into RNALater (Ambion) and stored at -80°C for analysis by PCR. RNA extraction and Taqman® qRT-PCR was performed on experimental kidney samples using the methods described above for amplification of ISAV segment 7, 8 and ELF1a. Samples were also processed for conventional RT-PCR detection of ISAV segment 8 which was previously described [5] and is in routine diagnostic use within our laboratory.

Application of qRT-PCR to the detection of ISAV in field samples during routine ISAV surveillance in Scotland

Field samples intended for ISAV diagnosis during the routine ISA surveillance programme conducted by FRS in Scotland were processed in parallel throughout a nine month period in 2004, using both the RT-PCR method and qRT-PCR methods reported in this study. During this period the presence of ISAV was suspected in Scotland, although it was not attributed as the cause of clinical disease [15]. During this investigation a total of 279 kidney and 87 gill tissue samples were processed for ISAV diagnosis by both qRT-PCR and conventional RT-PCR.

RESULTS

Development of Taqman real-time RT-PCR method

Validation of reaction efficiencies and relative quantification

The amplification efficiency graphs obtained from serial dilutions of ISAV-positive fish tissue are indicated in Figure 1. Each of the ISAV assays exhibited comparable efficiencies with that of ELF1α as indicated by them exhibiting a difference in slope (ΔS) of <0.1 [16]. This indicates that the ELF1α endogenous control is a suitable reference for the relative quantification of ISAV over the range of starting concentrations used in this study. For each reaction series, there was also a high correlation between cycle number and dilution factor (R^2 =1.0 in all cases) and slope values approached the optimum theoretical value of -3.32, indicating efficient amplification of all assays. Primer and probe concentrations were optimised at 900 nM and 250 nM respectively and no loss in sensitivity was recorded by reducing the total reaction volume to 25 µl.

Sensitivity

The segment 8 Taqman® qRT-PCR assay consistently detected ISAV at a log higher dilution that the segment 7 Taqman® qRT-PCR assay which itself consistently detected ISAV at a log higher than the conventional RT-PCR (data not shown). In addition, very faint products were observed nearing the endpoint of detection for conventional RT-PCR which required experience in their interpretation.

Specificity

Both segment 7 and 8 assays yielded no detectable product when using cDNA derived from viruses other than ISAV. Virus identified in field samples during 2004 proved to be of the HPR0 type, which demonstrated efficient detection of variants of ISAV [15].

Application of relative quantification to the detection of ISAV in experimentally infected fish

Experimental infection of salmon with ISAV

The mortality obtained in the unsampled tanks used to monitor the efficacy of the experimental infection is shown in Figure 2. Mortality commenced on day 11 post-infection and all fish in the ISAV infected group were dead by day 20. All dead fish showed clinical signs consistent with ISA disease which included pale gills, darkened livers and the presence of ascites. No mortalities were recorded in the control tanks throughout this period.
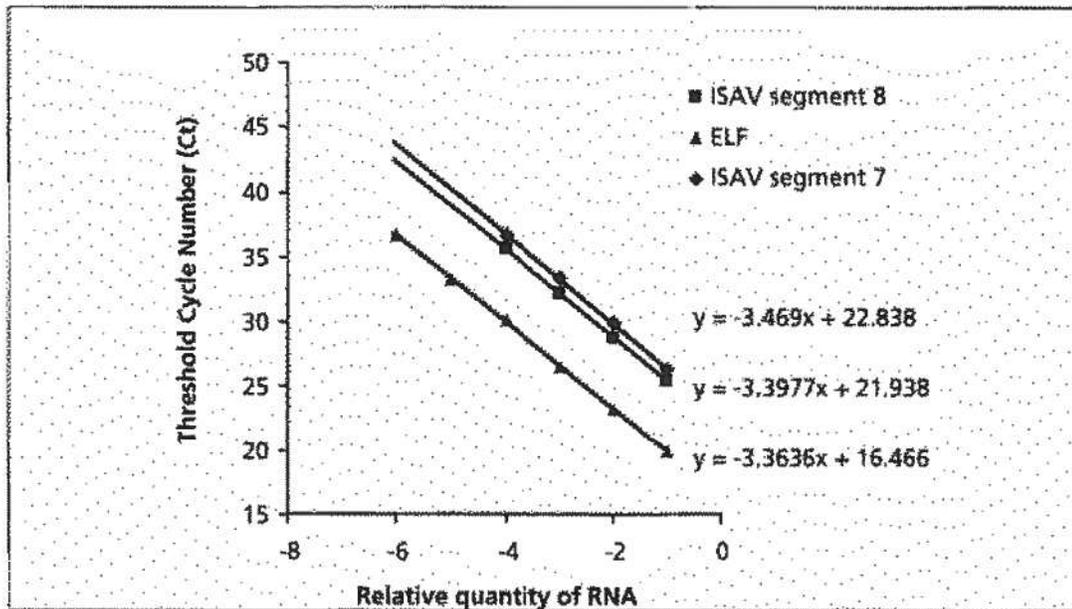
Fig. 1: Standard curves obtained by plotting the relative dilution of RNA versus the cycle number required to elevate the fluorescence signal above the threshold for the ISAV Segment 7, 8 and ELF assays. Equations were derived from linear regression.
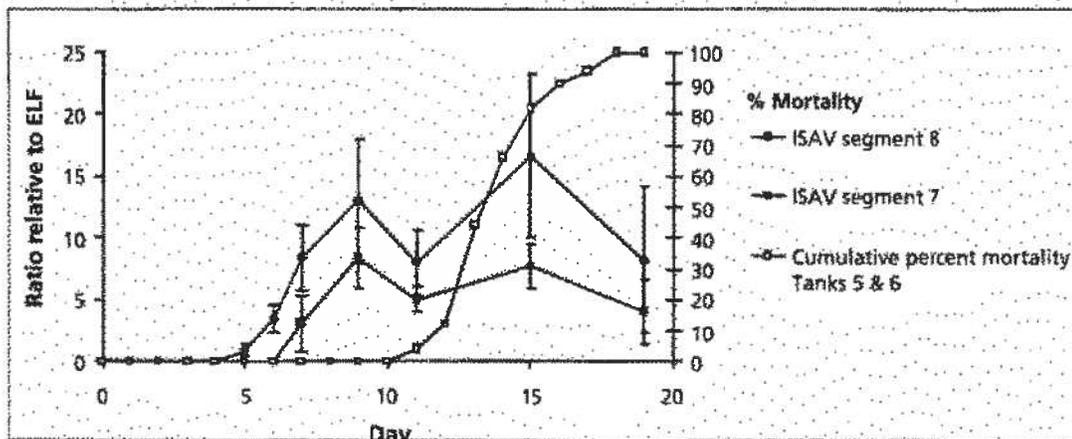

Fig. 2: **Graph indicating the mean level of ISAV segment 7 and 8 detected at different time-points following the experimental infection of Atlantic salmon with ISAV.** Mean levels were derived from four fish sampled at each time-point and represent the level (in arbitrary units) of each ISAV gene expressed as a proportion of the endogenous ELF-1α control signal detected in the same sample. Error bars indicate the standard error of the mean. The mean cumulative percent mortality obtained in un-sampled ISAV challenge reference tanks is indicated.

Sampling and comparative PCR analysis

Results obtained from the qRT-PCR analysis are detailed in Figure 2. The relative signal corresponding to target ISAV RNA was normalized to that obtained with the ELF1α endogenous control in each case. Values were expressed in arbitrary units. This calculation was performed according to the comparative Ct method described in the literature supplied with the ABI 7000 Sequence Detection System. All samples tested positive for ELF1α indicating efficient RNA extraction and reverse transcription in all cases. All fish testing positive for ISAV segment 8 also tested positive for ISAV segment 7. At day 1 post infection, two of the four fish sampled tested positive for ISAV, but at all subsequent time-points all fish tested positive. The mean values obtained for segment 8, however, were consistently higher that those obtained for the segment 7 assay, when expressed as a ratio to all ELF1α (Fig. 2). No contamination was recorded in any of the control fish sampled or in any of the RNA extraction (A), reverse transcription (B), real-time or conventional PCR controls (C) performed as described above.

Results obtained on the same sample set using a conventional RT-PCR targeting ISAV segment 8 corresponded with those obtained with the qRT-PCR methods, with the same two fish testing negative. In many of the positive cases, however, very weak products were observed on conventional agarose gels, which required experienced interpretation. In some cases non-specific products were also observed alongside the expected 155bp product.

Application of relative quantification to the detection of ISAV in field samples during routine ISA surveillance in Scotland

Two hundred and seventy nine kidney samples (each a pool of up to five fish) were screened for the presence of ISAV using Taqman® qRT-PCR assays targeting segments 7 and 8 in addition to conventional RT-PCR which also targets segment 8. Six samples were identified as positive using the conventional RT-PCR. All six samples were also positive using both ISAV segment 7 and 8 Taqman® qRT-PCR assays. qRT-PCR assays for segment 7 and 8 identified a further 11 and 12 positive samples, respectively (corresponding to 17 individual samples), which tested negative by conventional RT-PCR. Of these, six samples were positive using both assays, but five and six samples were positive using only the segment 7 or segment 8 assay respectively. Higher relative quantities of ISAV segment 7 and 8 were identified in samples testing positive for conventional RT-PCR as opposed to those testing negative for RT-PCR and positive by either one or both of the Taqman® assays (Figure 3). Interestingly, some of the values obtained from field samples were of a similar magnitude to those obtained during the course of clinical infection under experimental conditions.

During surveillance for ISAV, gill samples were also taken from some fish. Of a total of 87 fish samples processed, 41 yielded positive results using conventional RT-PCR and both segment 7 and segment 8 Taqman® qRT-PCR assays. No samples were positive using only conventional PCR. Seventeen samples were positive using both segment 7 and segment 8 assay but were negative using the conventional RT-PCR assay. Three samples were positive using the segment 7 Taqman® qRT-PCR assay only and a further 12 samples were positive using only the ISAV segment 8 Taqman® qRT-PCR. Relative quantities of virus detected are indicated in Figure 4. Samples which were positive using all methods yielded high relative quantities of virus. For those which tested negative using conventional RT-PCR, but positive using

both Taqman* qRT-PCR assays, low relative values were obtained for both segment 7 and segment 8 assays. Similarly samples testing positive using only one of the qRT-PCR assays also yielded low relative values.
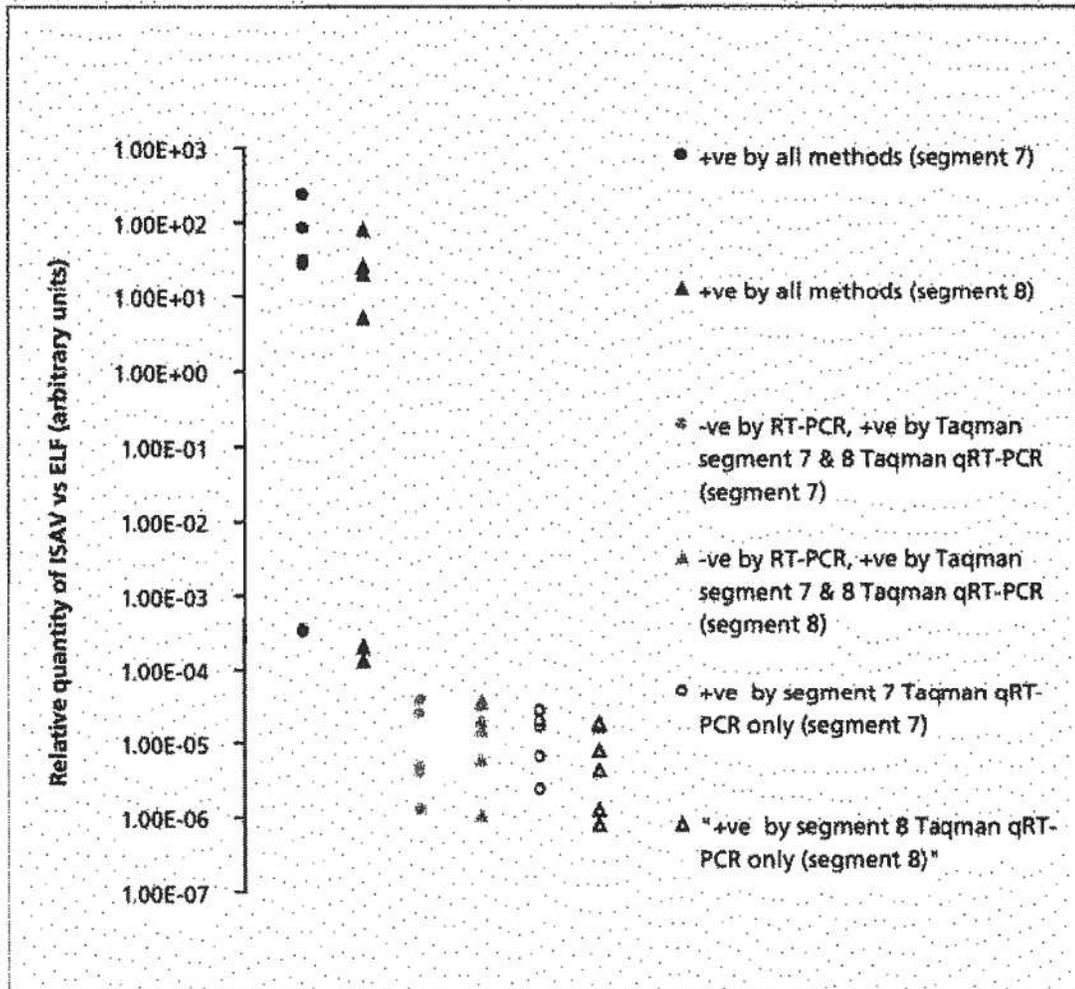

Fig. 3: **Relative levels of ISAV segment 7 and 8 detected in field kidney samples analysed during routine surveillance for ISAV in Scotland during 2004.** ISAV Levels obtained using Taqman* segment 7 (indicated by circles) and segment 8 (indicated by triangles) semi-quantitative qRT-PCR assays and expressed as a proportion of the endogenous ELF-1α control signal detected in each sample are detailed. Results are indicated according to the number of the three diagnostic tests (RT-PCR, Taqman* qRT-PCR segment 7, Taqman* qRT-PCR segment 8) generating positive results in each case.
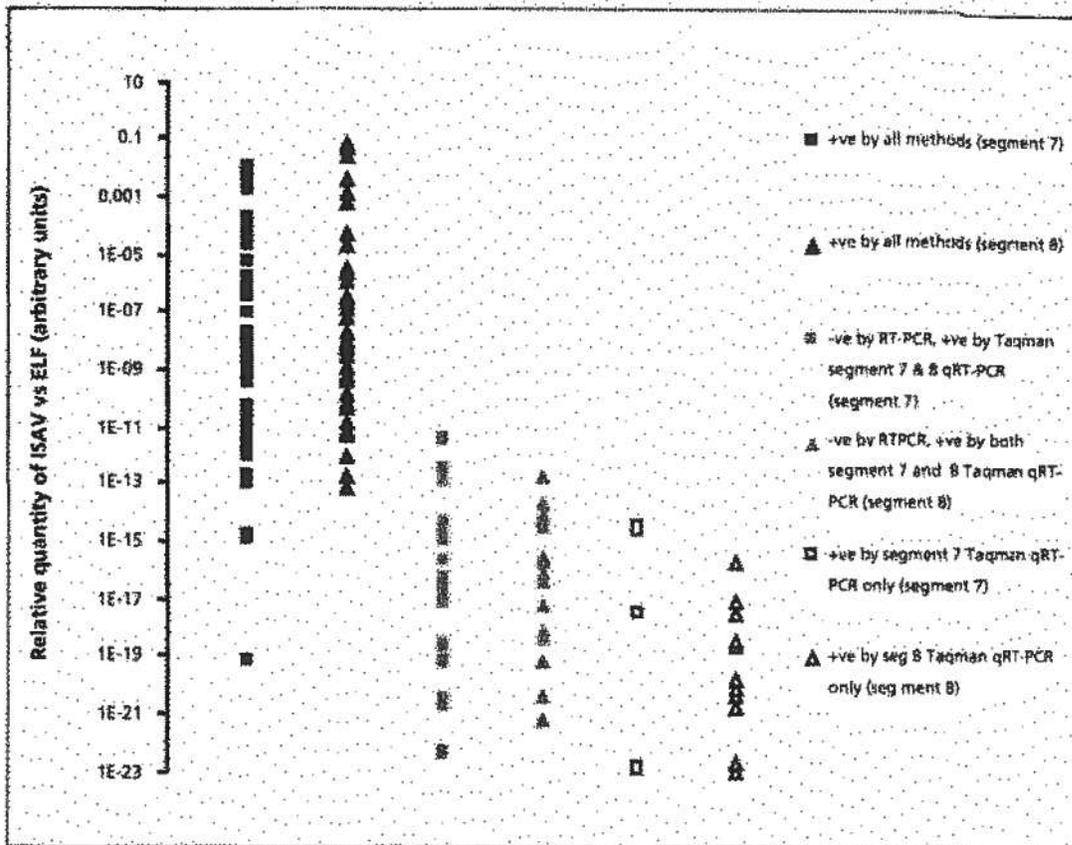
Fig. 4: **Relative levels of ISAV segment 7 and 8 detected in field gill samples analysed during routine surveillance for ISAV in Scotland during 2004.** ISAV Levels obtained using Taqman® segment 7 (indicated by squares) and segment 8 (indicated by triangles) semi-quantitative qRT-PCR assays and expressed as a proportion of the endogenous ELF-1α control signal detected in each sample are detailed. Results are indicated according to the number of the three diagnostic tests (RT-PCR, Taqman® qRT-PCR segment 7, Taqman® qRT-PCR segment 8) generating positive results in each case.

DISCUSSION

The results presented describe two independent highly sensitive and specific two-step Taqman® qRT-PCR methods for the detection of ISAV in Atlantic salmon tissues. Both methods proved to be more sensitive than the conventional RT-PCR method in routine use for ISAV diagnosis [5]. In addition, the application of semi-quantitative Taqman® qRT-PCR to ISAV diagnostics offers a number of advantages associated with this technology including an increased sensitivity, enhanced specificity, quantitative expression against endogenous controls, a lack of subjectivity in the interpretation of results, speed of processing and contamination control.

The segment 8 qRT-PCR assay was shown to be more sensitive than the segment 7 assay during initial experimental dilution series experiments. This was supported by the consistent detection of higher levels of segment 8 in experimentally infected fish during a clinical outbreak of ISA disease and the fact that more field kidney and gill

samples were positive by the segment 8 assay only than by the segment 7 assay only. Since the assays displayed similar theoretical efficiencies, such a finding is most likely related to the differential expression of segment 7 and 8 segments throughout the viral infection cycle. Both segments 7 and 8 of ISAV have been shown to encode two proteins, based on removal of an intron from the primary transcript [17] or from overlapping open reading frames, respectively [18]. Segment 8 has been shown to encode a major structural protein [19], while segment 7 has recently been demonstrated to encode at least one protein which mediates the host interferon defence system [20]. An increased abundance of segment 8 mRNA generated during the viral life cycle may thus explain the apparent improved sensitivity of the Taqman® segment 8 assay.

Both Taqman® assays performed at least as well as the RT-PCR segment 8 assay in the analysis of experimentally infected fish. This was perhaps not surprising since the fish had been injected with a relatively high dose of ISAV which, based on the severity of the challenge, was actively replicating. Analysis of serial dilutions, however, suggested an increased sensitivity of the Taqman® assays. This increased sensitivity may be related to the specificity and sensitivity of Taqman® chemistry which permits additional cycles of PCR to be conducted (n=45) when compared to conventional PCR (n=35). Furthermore, the generation of the very small amplicons in both ISAV Taqman® assays may contribute to the improved sensitivity observed. Improved sensitivity of both Taqman® assays over conventional RT-PCR was also demonstrated during evaluation of both kidney and gill samples collected as part routine diagnostic surveillance of Scottish salmon aquaculture during 2004. Indeed, many samples testing negative by conventional PCR tested positive by both segment 7 and segment 8 Taqman® assays. However, the relative quantity of ISAV detected was lower than in those samples which tested positive by conventional RT-PCR. In these cases, the agreement of the independent Taqman® assays, coupled to the detection of signals beyond the apparent detection threshold for conventional RT-PCR strongly supports the fact that these were indeed true positive samples. In some cases, samples were negative by conventional RT-PCR and positive by only one of the segment 7 or 8 Taqman® assays. In all these cases, relative levels of ISAV detectable were also very low. The demonstrated difference in sensitivity of the segment 7 and 8 assays, which is probably related to differences in viral gene expression could account for such differences and supports the view that the results observed are not due to a lack of assay specificity.

The specificity of detection using Taqman® chemistry is significantly improved as compared to either conventional RT-PCR or real-time PCR based on chemistry such as SYBR green. Indeed, detection of Taqman® PCR products is dependent on the annealing of highly specific probes. Experience in our laboratory and in other studies has shown the method to be capable of the absolute discrimination of PCR products based on the presence of a single nucleotide mismatch in the probe binding region. Such specificity raises the prospect of false negatives using a single probe, should mutation occur within this region. To reduce the likelihood of false negatives, probes in this study were designed from absolutely conserved regions based on alignments of all known ISAV sequence types. The parallel use of two independent assays in this study provides further confidence in the characterisation of true negatives and adds significant power to the detection of rapidly mutating organisms such as RNA viruses.

The development of a quantitative method for ISAV diagnosis using the ELF1α endogenous control provides a significant advantage over the conventional RT-PCR method in ensuring quality control of RNA and subsequently generated cDNA quality and quantity. The elongation factor 1 alpha gene has been previously demonstrated to

be stably expressed between different individuals, and as such represents a suitable calibrator for semi-quantitative real-time PCR [21]. ISAV load expressed as a function of ELF1α is thus a measure which can be compared across individuals sampled at different times. In a diagnostic context such information can be used to express confidence levels in positive results obtained and as a basis to predict the likelihood of other tests (e.g. conventional RT-PCR or virus culture) also yielding positive results. Results might also be used to predict the clinical status of fish once a more comprehensive understanding of the relationship between viral RNA levels and pathology emerges. Such a method thus offers considerable scope both for improving the understanding and interpretation of PCR results, generating more information than a simple positive/negative result obtained using conventional RT-PCR. Indeed, within the current study of ISAV experimentally infected fish, we have demonstrated the range of signals expected in kidney tissues throughout a clinical disease outbreak. Results from kidney samples obtained in field samples during 2004 [15] were in some cases of a similar magnitude to these values, although in others relative values were significantly lower. Such knowledge might in future assist us in identifying carrier fish populations and those in which clinical disease is expected. In this case, however, further characterization of detected virus identified it as an HPR0 ISAV subtype, the relevance of which to development of ISA disease is not fully understood.

Taqman* qRT-PCR does not require any post-PCR processing and in particular does not require the subjective interpretation of weak products visualised on agarose gels. As such, interpretation of its results is more objective and less variable and suited to implementation in routine viral diagnostic testing. The lack of post-PCR processing also reduces the time taken to complete the method (approximately four to five h) and minimises the potential for obtaining false positives due to cross contamination. The Taqman* method also replaces dTTP with dUTP during PCR amplification. Subsequent PCR mixes are treated with the enzyme uracil N-glycosylase (UNG) which specifically degrades PCR products from previous amplifications in order to reduce the risk of amplicon carry-over resulting in sample contamination [22].

In conclusion, two highly sensitive and specific Taqman* qRT-PCR assays targeting ISAV segment 7 and segment 8 have been developed and evaluated. Use of an internal endogenous control ELF1α allows semi-quantitative expression of viral transcript levels. The application of Taqman* technology to ISAV diagnosis offers a number of significant advantages over conventional RT-PCR and lends itself well to implementation within a routine viral diagnostic environment.

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Dr. M. Snow, FRS Marine Laboratory, 375 Victoria Rd, Aberdeen, AB11 9DB, U.K.
E-mail: M.Snow@marlab.ac.uk