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Epidemiological Investigation of Infectious Salmon Anaemia (ISA) Outbreaks in Norway 2003-2005

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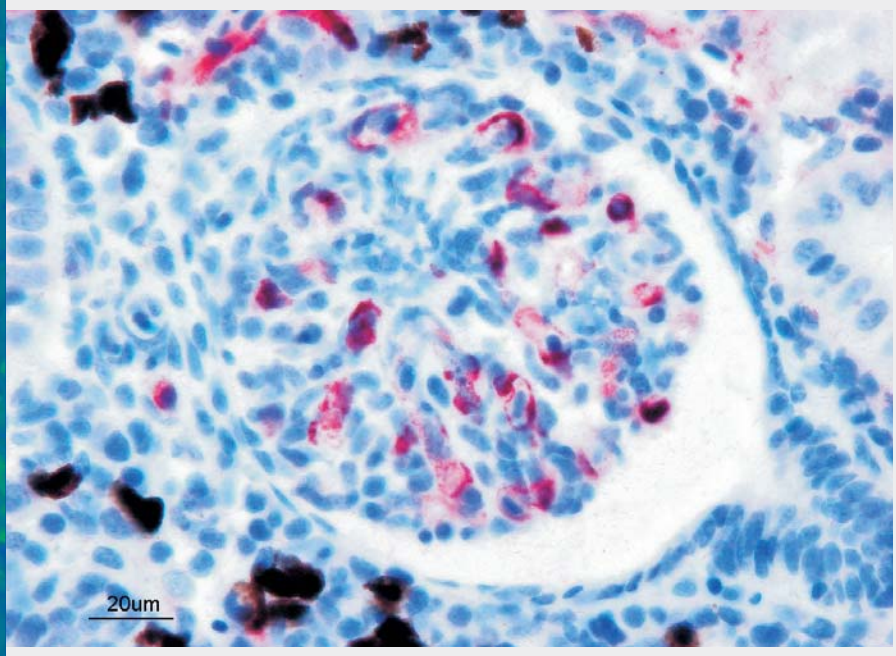

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Epidemiological Investigation of Infectious Salmon Anaemia (ISA) Outbreaks in Norway 2003-2005

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Contents

Norsk sammendrag

Veterinærinstituttet(VI) har oppsummert epidemiologiske rapporter fra 32 utbrudd av infeksiøs lakseanemi (ILA) i Norge i perioden 2003-2005. Detaljer om ILA utbruddene i 2005 er presentert i Appendiks I. VI har tidligere oppsummert epidemiologiske rapporter om ILA utbrudd i 2003 og 2004 (Lyngstad TM *et al*., 2005a; Lyngstad TM *et al*., 2005b).

Epidemiologisk informasjon fra utbruddene ble samlet inn av Mattilsynets distriktskontorer i henhold til direktiv 93/53/EEC art. 8, og ble sammen med resultatene fra genotypingen brukt til å teste mulige assosiasjoner mellom utbrudd med hensyn på nærhet til andre lokaliteter, kontaktnettverk, og felles smoltleverandør. Trettien av ILA virus (ILAV) isolatene ble genotypet av VI mens ett av isolatene ble identifisert og genotypet av Nylund *et al*. (2007).

ILA utbruddene var spredd langs hele kysten, og viste et sammensatt klinisk bilde. Klinisk ILA synes å utvikle seg langsomt, noe som indikerer at en lokalitet kan være smittet i flere måneder før diagnosen blir stadfestet. I gjennomsnitt hadde fisken stått på sjølokaliteten i 14 måneder før ILA ble stadfestet. Smolt fraktes langs hele kysten (opptil 1800 km) og alle lokalitetene mottok smolt via brønnbåt. Opptil 12 anløp med smolt ble rapportert på en lokalitet. Førtiseks ulike smoltleverandører levert smolt til de 32 lokalitetene med ILA utbrudd. Trettiseks av dem hadde levert hver til ett utbrudd, en hadde levert til 6 ulike ILA utbrudd.

Genotypisk karakterisering viste at isolatene fra de 32 utbruddene grupperte i de tre foreslåtte genogruppene G1, G2 og G3 (Nylund *et al*., 2003). De ulike genogruppene var spredd langs hele kysten og alle genogruppene var representert i utbrudd fra hvert år i perioden.

Analysen viser en assosiasjon mellom ILA utbrudd med hensyn på nærhet og felles kontaktnettverk. Dette er forenlig med horisontal smitteoverføring. Vi fant ikke noe signifikant mønster relatert til smoltleverandører.

Summary

Epidemiological information was summarized from 32 outbreaks of infectious salmon anaemia (ISA) in Norway 2003-2005. Details about ISA outbreaks in 2005 are presented in appendix I in this report. The National Veterinary Institute has earlier summarised case reports about ISA outbreaks in 2003 and 2004 (Lyngstad TM *et al*., 2005a; Lyngstad TM *et al*., 2005b).

Virus isolates from the outbreaks in 2003-2005 were genotyped, and postulated associations between outbreaks due to risk factors were assessed. The ISA outbreaks were distributed along most of the Norwegian coast and showed a variable clinical picture. The virus genotypes clustered into three genogroups, and tended to scatter in time and along the coast. ISA outbreaks matched for the risk factors "proximity" or "contact", shared a significantly higher number of mutual genogroups than expected. For the risk factor "smolt supplier", corresponding genogroups appeared in seven out of 12 matched pairs, which was not significant.

In conclusion, genotyping of virus isolates from ISA outbreaks supports associations between adjacent outbreaks. This is consistent with horizontal transmission. The present study failed to find patterns of genogroups related to smolt suppliers or brood fish companies.

Introduction

Infectious salmon anaemia (ISA) is a viral disease of Atlantic salmon (*Salmo salar* L.) which was first diagnosed in Norway in 1984 (Thorud and Djupvik, 1988). Since then, the disease has been described in Atlantic salmon in Scotland, Canada, and the Faroe Islands. The ISA virus (ISAV) has also been recorded from Coho salmon (*Oncorhynchus kisutch*) in Chile (Cipriano and Miller, 2003). ISA was made notifiable as a List B disease in Norway in 1988. Within the EU, ISA is classified as a non-exotic disease in Council Directive 2006/88/EC. A total of 438 outbreaks have been reported in Norway during the time period from 1984 to 2005. The yearly number of outbreaks peaked in 1990 with a total of 80 cases. In the late 1980ies and the early 1990ies, the Norwegian veterinary authorities implemented several measures such as a ban on using non-disinfected sea water in hatcheries and movement of fish from one sea water site to another. Furthermore, compulsory health certificates for aquaculture farms, regulations on disinfection of waste water from slaughterhouses and processing plants were implemented (Thorud and Håstein, 2003). Since 1993, the annual incidence of ISA has varied between one and 20 (Figure 1).

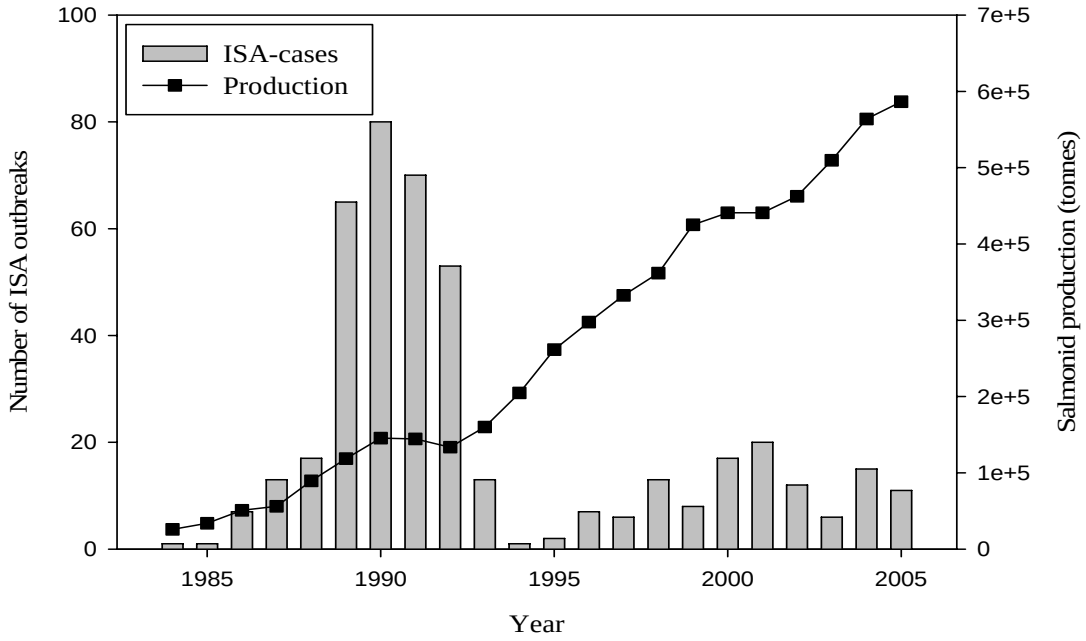


Figure 1. Verified ISA outbreaks and the salmonid production in Norway from 1984 to 2005 (source: Directorate of Fisheries and the National Veterinary Institute, T. Håstein).

ISAV has a segmented genome with eight different segments. The haemagglutinin-esterase (HE) gene is located on segment 6 and codes for the main surface glycoprotein in ISAV. It contains three different domains: an N-terminal portion with some sequence variation, which constitutes the surface-exposed region, a transmembrane domain, and a more conserved C-terminal part. A short hyper variable region (HPR), characterized by variable amino acid deletion patterns, is located right upstream of the transmembrane region. The database includes a large number of sequences for the HE gene from different isolates. Even though the sequence variation is not substantial for the European isolates, a sub classification of the European isolates into three groups (G1, G2 and G3) has been proposed, based on the most varied part of the HE gene (the 5'-flanking region). This classification may form a basis for the assessment of kinship between ISAV isolates (Nylund *et al*., 2003). The HPR-region varies significantly between related isolates due to deletions, and is not suited as an indicator of kinship. A grouping of ISAV isolates into HPR groups has however, been made, with the full-length HPR (without deletions) as the HPR0 (Nylund *et al*., 2003; Nylund *et al*., 2007). HPR0 appears to be associated with low virulence, while every other type of HPR has been isolated in association with disease outbreaks (Cunningham *et al*., 2002; Mjaaland *et al*., 2002; Nylund *et al*., 2007). The number of different HPRs being detected is increasing as there seems to be a potential for a great variety of HPR0-deletions.

Epidemiological studies in the 1990ies indicated that ISA disease was most often transmitted by movement of infected live salmon, animal waste, or effluents (blood or somatic cells or organic particles infected with the ISA agent). The risk of an ISA outbreak was associated with the site's proximity to infectious sites and slaughterhouses (Jarp and Karlsen, 1997; Vågsholm *et al*., 1994). Similar conclusion was reached in a study from New Brunswick (McClure *et al*., 2005). A recently developed stochastic model quantified the relative importance of seaway distance and contact network as risk factors for horizontal transmission between sites (Scheel *et al*., 2007). An association between number of vessel visits moving fish between sites and site contamination has also been demonstrated (Murray *et al*., 2002). It is commonly believed that the virus is not transmitted vertically (Cipriano and Miller, 2003). However, Nylund *et al*. (2007) suggest that some sort of vertical or transgenerational transmission may occur. These authors used genotyping of the haemagglutinin-esterase gene of the ISAV in conjunction with data on the origin of smolt, eggs and broodfish in an attempt to trace the origin of the virus from different outbreaks of ISA in Norway.

The aim of the present study was to summarize ISA outbreak case reports in Norway 2003-2005, present results from genotyping of virus isolates from the outbreaks, and to test if the genotyping supports associations between ISA outbreaks and risk factors for transmission.

Materials and methods

Case definition

An ISA outbreak is defined in accordance with The Contingency Plan for Control of ISA in Norway (Anon., 2004). A positive diagnosis is based on clinical signs, post mortem findings, and laboratory investigations.

Data collection

The case reports from the individual ISA outbreaks in 2003-2005 were gathered by the Local District Offices of the Norwegian Food Safety Authority in accordance with EU Directive 93/53/EEC Art. 8.1. The reports provided information on diagnostic data, fish health, stock and management. The recording was not standardized until 2005 when a questionnaire was designed for the data collection. The level of detail therefore differs between years in the study.

Data on broodstock origin was compiled from Nylund *et al*. (2007) (Table 3).

Genotyping

RNA was isolated from head kidney of two individuals from each outbreak with Qiagen RNEasy mini kit or on an automated NucliSens® easyMAGTM from BioMerieux following the protocol for off-board lysis. RT-PCR with the primers klon1EGFPF1 & klon1EGFPR1 (Mjaaland *et al*., 2002), and the alternative primer set: ILAHA1F 5'-GCAAAGATGGCACGATTCATA-3' & ILAHA1R 5'-AGCAACAGACAGGCTCGAT-3' and Superscript III/Qiagen HotStar enzyme was performed in order to transform RNA into cDNA and propagate the ISAV HE gene. The PCR products were purified using a Qiagen PCR purification kit or Qiagen Gel Extraction kit, depending on the quality of the PCR product. A sequencing-PCR using a BigDye Terminator Sequencing kit (Applied Biosystems) and the primers listed above and also internal primers Seg6U 5'-GGAATCTACAAGGTCTGCATTG-3' and ILAHA2R 5'-TAGGAACAGAGCAATCCCAAA and 4 internal primers described previously (Devold *et al*., 2001) was then performed . The resulting products were run through a 3100Avant Genetic Analyzer (ABI) or Megabace 1000 (AME BioScience). The sequences were then analysed using the Sequencher 4.1.4 software from GeneCodes, and the amino acid configuration of the hyper variable region (HPR) was examined. BioEdit (©T. Hall, Dep. Microbiol., North Carolina State University) was used to create an alignment of the 5' flanking region of HE genes (c. nucleotide 50-950) from the various outbreaks, as well as representative isolates which had previously been sequenced from genogroups G1, G2 and G3 (Nylund *et al*., 2003). Phylogenetic analysis was carried out using the PHYLIP Package, version 3.65 (Joe Felsenstein, Department of Genome Sciences, University of Washington, Seattle, Washington, USA, http://evolution.gs.washington.edu/phylip.html), and the phylogenetic tree was visualised using TreeView (Win32) version 1.6.6 (Roderick D. M. Page, Division of Environmental and Evolutionary Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK, http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Associations between genogroups and risk factors

An exploratory retrospective analysis was conducted to test if the geno-grouping supported associations between ISA outbreaks and the risk factors; 1) proximity between outbreaks, 2) possible contact between outbreaks, or 3) the sharing of smolt suppliers. Starting with the last occurring outbreak in 2005 we sequentially moved backwards in time through the list of outbreaks, and matched pairs if they conformed to a set of rules that complied with a possible association to a given risk factor. A common rule for this procedure was that a matched pair of outbreaks did not have their date of outbreak verification more than 12 months apart. If this criterion was met, outbreaks were matched for "proximity" as a risk factor if they were located within 10 km seaway distance of each other, and outbreaks were matched for "contact" as a risk factor if they were registered under the same concession identification. Geographical coordinates and concession identifications were compiled from the aquaculture licence register of The Directorate for Fisheries (www.fiskeridir.no) in September 2004. Finally outbreaks were matched for "smolt supplier" as a risk factor if they shared one or more suppliers. Thus, a sequence of 7, 5 and 12 matched pairs of outbreaks were obtained for the three risk factors, respectively. For each pair the genogroups of the two outbreaks in matched pairs were compared and assigned a success (S) if they were alike or a failure (F) if they were different (Table 4).

A binomial test was used to test if there was a significantly higher number of S's (one-sided) than expected by chance for each of the three risk factors, unconditionally. The expected probability of an S was set to 0.40 which corresponds to the number of S's (n=213) divided by the total number of possible outcomes (N = 528) when two genogroups are randomly picked from the distribution of genogroups given in Table **3**. Note that the probability of an S outcome has been adjusted with regard to the two outbreaks from where two different genogroups have been demonstrated.

Results

Case characteristics 2003-2005

In the period 2003-2005 a total of 32 outbreaks of ISA were reported by the Norwegian Food Safety Authority. Twenty-three of the outbreaks were classified by the Norwegian Food Safety Authority as primary outbreaks and nine as secondary. Case reports from all primary outbreaks and four secondary outbreaks were received in the present study. The ISA outbreak sites were distributed along most of the Norwegian coast (Figure 2). On average the fish were reared on sea sites for 14 month before ISA was verified (Figure 3).

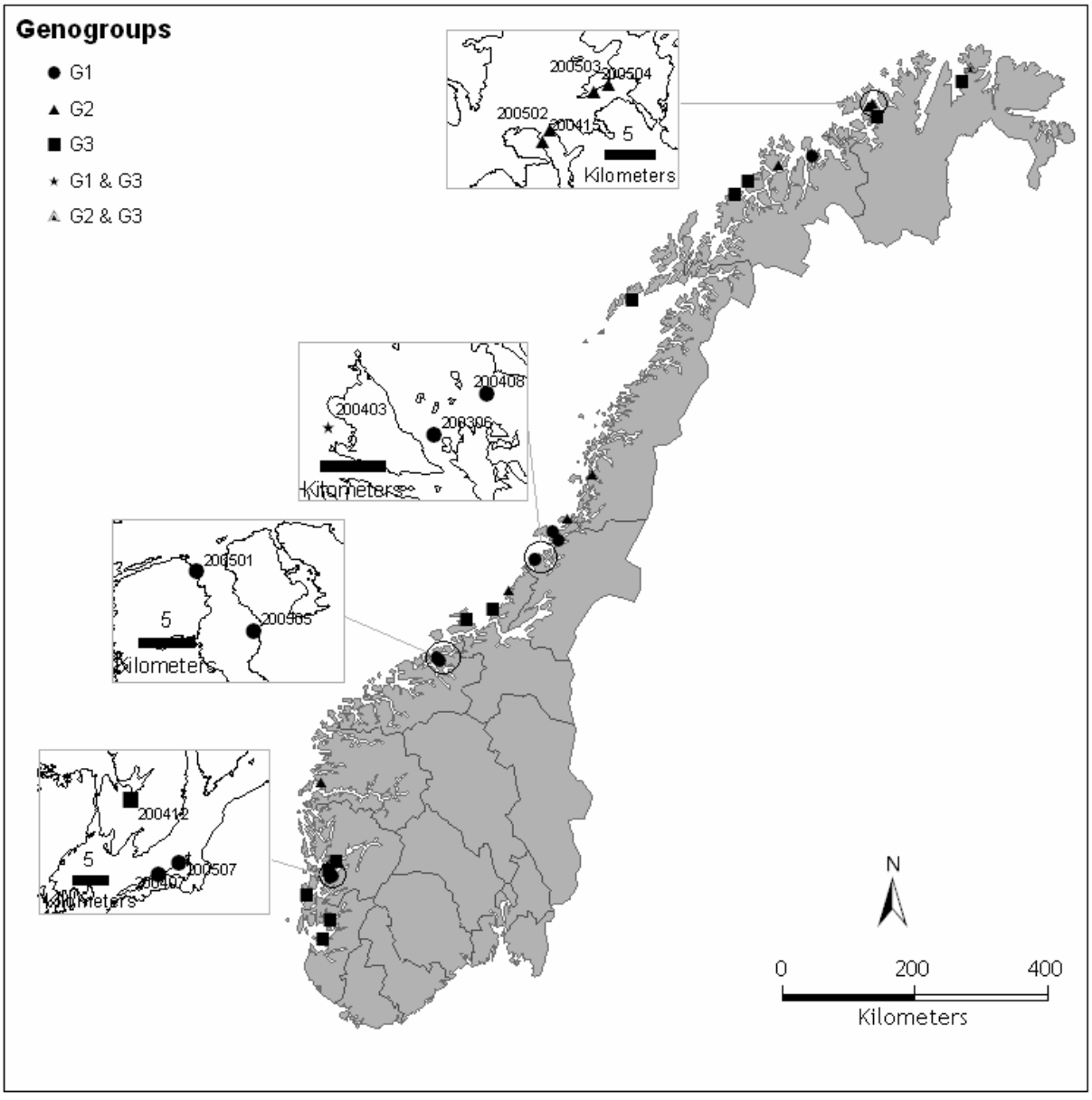


Figure 2. Verified ISA outbreaks in Norway in 2003-2005 denoted according to genogroups. Four areas are enlarged (in frames) due to proximity between outbreak sites.

Table 1. Seasonal distribution of ISA outbreaks in Norway throughout the years 2003-2005

Season	Number of outbreaks
January - March	5
April – June	12
July - September	7
October - December	8

All outbreaks in 2003-2005 were on sea water sites rearing Atlantic salmon. Two of the outbreak sites reared rainbow trout in addition, but no clinical signs of ISA were recorded on this species. The ISA outbreaks occurred in all seasons with a peak during April - June (Table 1).

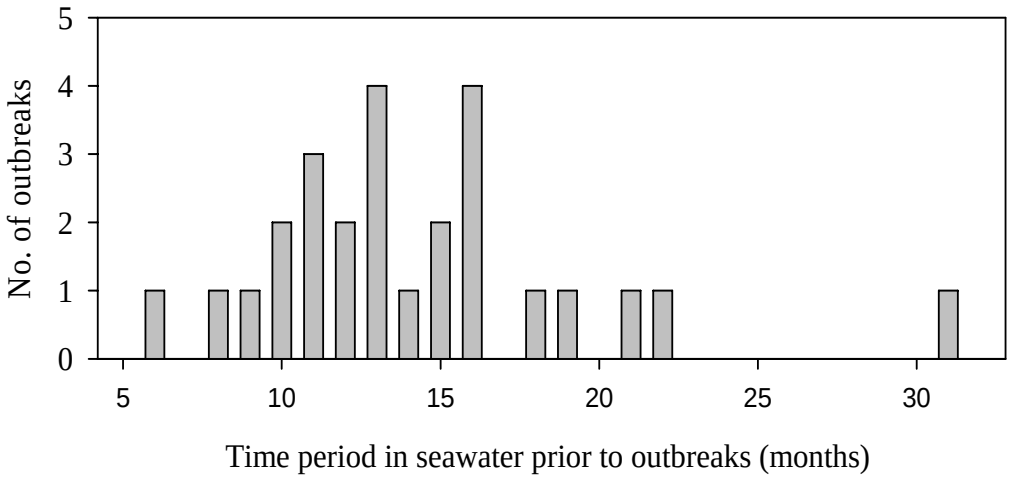


Figure 3. Frequency distribution of the time period that Atlantic salmon had been reared in seawater sites prior to outbreak verification.

Nineteen of the 32 outbreak sites reported clinical signs consistent with ISA. Infectious pancreatic necrosis was reported from 14 of the 32 outbreak sites before ISA was diagnosed. Winter ulcer and parvicapsula was reported from five sites each. Cardiomyopathy syndrome and heart and skeletal muscle inflammation was reported from four sites each. Pancreas disease was reported twice.

A total of 46 different smolt suppliers supplied smolt to the 32 sites experiencing ISA outbreaks. Thirty-six suppliers delivered to one outbreak, and one supplier delivered to six ISA outbreaks (Table 3). The mean number of smolts transferred to the different outbreak sites was 586,408 (min 228,900 – max 892,276).

All sites received smolts by well boat transport, and up to 12 visits were reported prior to an outbreak. Smolts on outbreak sites had been transported from 10 to 1800 km along the coast before sea transfer Table 3). Sites with ISA outbreaks were on average less than 5 km from the main coastal transportation route, but no transport was reported to have gone through an ISA control zone.

Genotyping 2003-2005

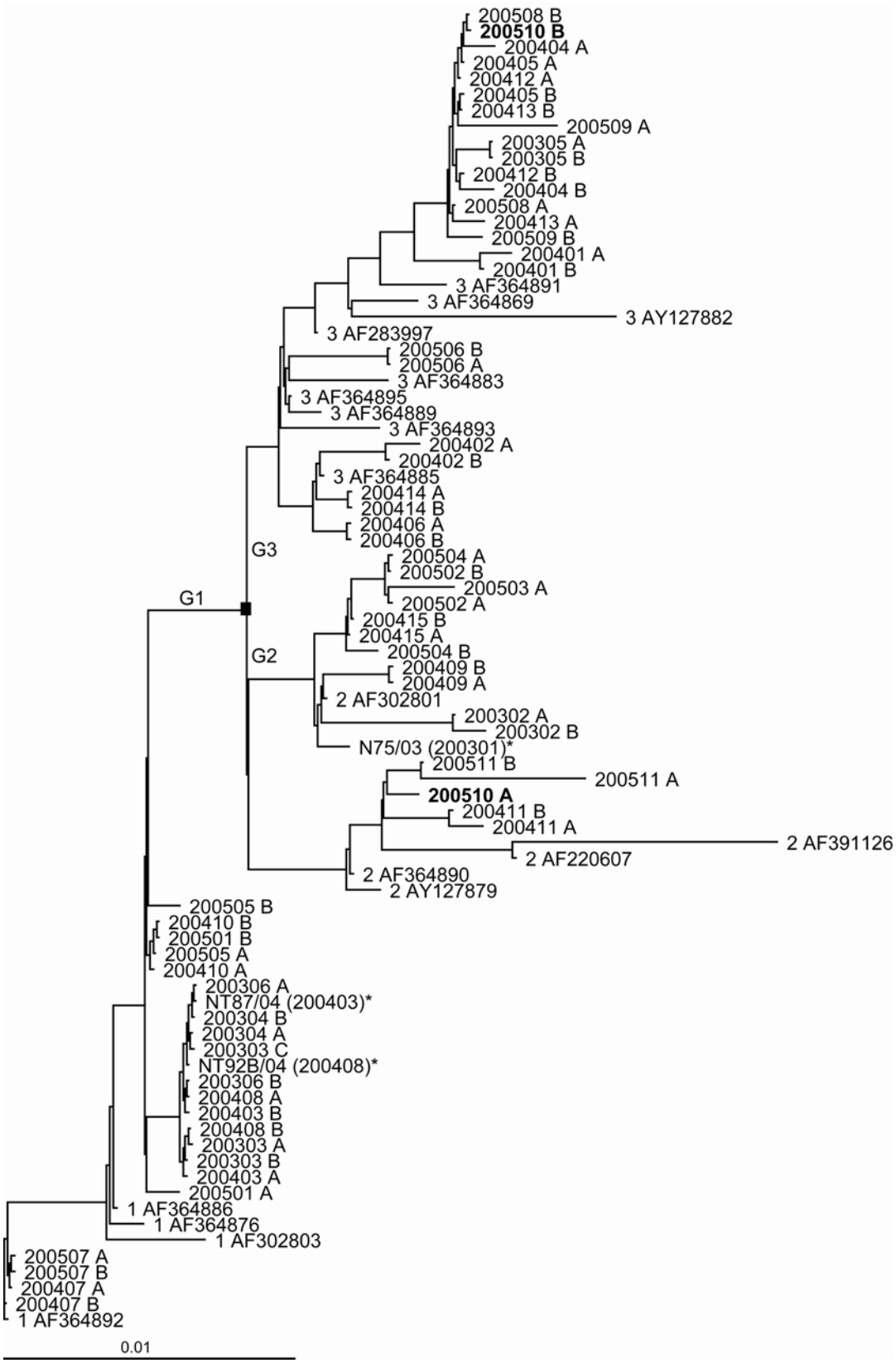
Thirty-one of the ISA virus isolates from the outbreaks in 2003-2005 were genotyped at the National Veterinary Institute. The genogroup from an ISA virus isolate from outbreak number 200301 was identified by Nylund *et al*. (2007). From outbreak number 200408 Nylund *et al*. isolated both G1 and G3, while the

National Veterinary Institute only isolated G1. Results from genotyping show variations in the HPR, whereas analyses of the more conserved 5` region support the clustering of European ISA virus isolates into three genogroups, G1-G3. These genogroups tended to scatter randomly along the coast and were represented in outbreaks from each of the three years in study (Table 3, Figure 4).

The ISAV HE gene has an open reading frame which varies in length from 1,161-1,233 nucleotides, depending on the length of the hyper variable region (HPR) which varies between 11 and 35 amino acids. The amino acid configurations in the HPR region were determined for two isolates from each outbreak. There was considerable variation between the different outbreaks with respect to their amino acid configuration in this region, and in total 15 different HPR configurations were found. In one case, outbreak number 200404, variation in the HPR region sequence was even found between two specimens from the same outbreak, even though the remainder of the sequence used for genotyping indicated close kinship between these two isolates (Table 2). On the other hand, in one case, 200510, the isolates A and B belonged to two different genogroups (G2 and G3, respectively), but showed identical sequences in HPR (Table 2, Figure 4). No isolate from the outbreaks showed the HPR0 sequence (European consensus sequence).

Table 2. Amino acid configuration in the HPR-region from selected ISAV-isolates from outbreaks in 2003-2005

HPR 0	T D V	K I	R	V	D	A	I	P	P	Q	L	N	Q	T	F	N	T	N	Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M			Geno-
Isolate	Aa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	G	V	A	group
200404 A	T D V	K	I	R	V	D	A												N	Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200404 B	T D V																							P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200405 A	T D V	K	I	R	V	D	A												N	Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200405 B	T D V	K	I	R	V	D	A												N	Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200412 A	T D V	K																								T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200412 B	T D V	K																								T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200509 A	T D V	K	I	R	V																			P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200509 B	T D V	K	I	R	V																			P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200413 A	T D V	K																								T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200413 B	T D V	K																								T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200305 A	T D V	K	I	R	V	D	A												N	Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200305 B	T D V	K	I	R	V	D	A												N	Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200508 A	T D V	K	I	R	V	D	A	I	P	P	Q								N	Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200508 B	T D V	K	I	R	V	D	A	I	P	P	Q								N	Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A	G3
200510 A	T D V	K	I	R	V	D	A	I	P	P	Q	L	N	Q	T																		F	I	S	M	G	V	A	G2
200510 B	T D V	K	I	R	V	D	A	I	P	P	Q	L	N	Q	T																		F	I	S	M	G	V	A	G3
200511 A	T D V	K	I	R	V	D	A	I	P	P	Q	L	N	Q	T																		F	I	S	M	G	V	A	G2
200511 B	T D V	K	I	R	V	D	A	I	P	P	Q	L	N	Q	T																		F	I	S	M	G	V	A	G2
200411 A	T D V	K	I	R	V	D	A	I	P	P	Q																				H	I	F	I	S	M	G	V	A	G2
200411 B	T D V	K	I	R	V	D	A	I	P	P	Q																				H	I	F	I	S	M	G	V	A	G2



* Sequence published by Nylund *et al*. (2007)

Figure 4. Phylogenetic tree of the ISAV-isolates from outbreaks in 2003-2005 constructed with the maximum likelihood method.

Table 3. ISA outbreaks in Norway 2003-2005 ordered chronologically

Outbreak no.	Sea site ID[a]	County	Geno-group	Brood-stock[b]	Smolt supplier ID[a]	Maximum transport (Km)
200301	10875	Nordland	G2[a]	C, D	11134, 13188, 11127	200
200302	10398	Sør-Trøndelag	G2	-	12745, 12217, 12672, 10412, 24096	300
200303	15657	Troms	G1	-	-	-
200304	12668	Nord-Trøndelag	G1	A, B	-	-
200305	13518	Troms	G3	-	11579, 10141	1,100
200306	12629	Nord-Trøndelag	G1	A, B	12415, 10412, 12745, 12672	200
200401	13057	Hordaland	G3	-	12103	10
200402	13083	Sør-Trøndelag	G3	-	12596, 13178	50
200403	12632	Nord-Trøndelag	G1	-	-	-
200404	19335	Finnmark	G3	-	10141, 10665	>1,800
200405	11193	Nordland	G3	B	10141, 13923	>1,100
200406	13794	Troms	G3	A, B, D	11333, 12269	>1,000
200407	13232	Hordaland	G1	A, B	13826, 11493	40
200408	19356	Nord-Trøndelag	G1&G3[a]	-	-	-
200409	10753	Troms	G2	A, B	11579, 12177, 10563, 10578	>1,300
200410	12715	Nord-Trøndelag	G1	A, B	12623, 13742, 12269	450
200411	11645	Sogn og Fjordane	G2		11678, 13152	75
200412	12110	Hordaland	G3		10141	20
200413	11574	Hordaland	G3		11589, 13637	36
200414	10790	Finnmark	G3	A	13140, 13191, 11180	600
200415	15517	Finnmark	G2	A, E	13140, 12719, 13482	1,500
200501	10214	Møre og Romsdal	G1	A, B	10221, 12898	56
200502	10836	Finnmark	G2	A, E	12719, 13140, 13482	>1400
200503	10616	Finnmark	G2	A	12474, 12719	>1100
200504	10834	Finnmark	G2	E	13140	>1400
200505	17495	Møre og Romsdal	G1	A, B	-	-
200506	12407	Sør-Trøndelag	G3	A	12992, 12430	14
200507	22095	Hordaland	G1	-	12041, 13823,	570
200508	11925	Rogaland	G3	-	10323, 12172	130
200509	10113	Rogaland	G3	B	11893, 10141, 12116	21
200510	13143	Finnmark	G2&G3	-	-	-
200511	14245	Nord-Trøndelag	G2	-	13 180	86

[a] Sea site ID as registered in the aquaculture licence register of The Directorate for Fisheries
[b] Nylund *et al*. (2007)

For the purpose of genotyping, the 5'-flanking end of the haemagglutinin-esterase gene was used. A phylogenetic analysis was performed on sequences from two specimens from each of the ISA outbreaks in 2003 to 2005, as well as on previously sequenced isolates obtained from the GeneBank database. The resulting phylogenetic tree is shown in Figure 4.

All the three genogroups were represented in the ISA outbreaks in 2003-2005 with 10 outbreaks belonging to G1, 10 outbreaks to G2, and 14 outbreaks to G3. For the outbreak 200510, the isolates belonged to two different genogroups (G2&G3). This was also the case for outbreak 200408 (G1&G3), although only one of these genogroups (200408 A, B & NT92b:G1) is included in our map. The sequence for NT92a available in GeneBank was too short to be included in our phylogenetic analysis, but is previously reported to belong to the G3 genogroup (Nylund *et al*., 2007).

Associations between genogroups and risk factors

Outbreak pairs that were matched for the risk factors "proximity" and "contact" all shared a mutual genogroup, which was a significantly higher number of successes than expected by chance for both risk factors. All pairs matched for the "contact" risk factor also matched for "proximity" (Table 4). For the "smolt supplier" risk factor corresponding genogroups appeared in seven out of 12 pairs that matched, which was not significantly higher number of successes than expected by chance (Table 4).

Table 4. Matched pairs of ISA outbreaks for the risk factors proximity (seaway distance < 10 km), contact (registration within the same concession) and smolt supplier (sharing one or more smolt suppliers). Common genogroups between pairs are denoted S, different genogroup are denoted F. Months apart refer to months between verification of outbreaks for the pairs

Matched Outbreaks	Months apart	Geno-group	Proximity	Contact	Smolt Supplier
200509 - 200412	9	G3, G3			S
200507 - 200407	11	G1, G1	S		
200505 - 200501	1	G1, G1	S	S	
200504 - 200503	<1	G2, G2	S	S	
200504 - 200502	1	G2, G2			S
200503 - 200502	1	G2, G2	S	S	S
200502 - 200415	3	G2, G2	S	S	S
200415 - 200414	<1	G2, G3			F
200412 - 200405	3	G3, G3			S
200410 - 200406	3	G1, G3			F
200409 - 200305	9	G2, G3			F
200408 - 200403	8	G1&G3, G1	S		
200405 - 200404	1	G3, G3			S
200404 - 200305	7	G3, G3			S
200403 - 200306	4	G1, G1	S	S	
200306 - 200302	5	G1, G2			F
200305 – 200303	3	G3, G1			F
Summary statistics binomial test					
N			7	5	12
Observed S			7	5	7
Expected S			2.8	2.0	4.8
P			0.002	0.011	0.16

Discussion and conclusion

In the present study genotyping of the haemagglutinin-esterase gene was used in conjunction with postulated associations between outbreak sites due to risk factors. Specifically, ISA outbreak sites were postulated to be associated if they were infected within a reasonably short time span, if they were located in proximity, if they were registered within the same concession, or if they shared one or more smolt suppliers. Outbreak sites thus associated were assessed with regard to kinship based on the genotyping.

All the ISAV isolates from the outbreaks in 2003-2005 grouped within three genogroups, G1, G2 and G3, which agrees with the geno-grouping first proposed by Nylund *et al*. (2003). There was no apparent geographic trend, nor any apparent time trend, with respect to the incidence of the different genogroups. In the retrospective analysis of the ISA outbreaks, pairs of outbreaks that were matched due to being located in proximity or that were registered within the same aquaculture concession, invariably belonged to the same genogroup (Table 4). The probability for this sequence of events occurring by chance is very low. Hence, we conclude that the observed genogroups support a common origin of the virus isolates from outbreaks that are located in proximity. This is consistent with a process involving horizontal transmission of the disease (Jarp and Karlsen, 1997; Vågsholm *et al*., 1994). Whether this is due to transmission through contact between outbreak sites, passive transmission via sea water, or some other local factor, is not discernable with the present data. Since all the pairs of outbreaks that were matched for the contact risk factor also were matched for the proximity risk factor, it is not possible to assign associations singularly to any of the two risk factors.

Pairs of outbreaks that were matched due to sharing smolt suppliers did not share genogroups significantly more often than expected by chance, although the observed number of successes was higher than expected. This means that we cannot conclude that there is significant evidence for a common origin of the virus isolates from outbreaks that share smolt suppliers. Nor can we rule out infected smolt being a risk factor for ISA outbreaks. However, it is noteworthy that for the most northerly cluster of four outbreaks (see Figure 2), which were all in close proximity and registered under the same company, there was no common smolt supplier that covered all of the outbreak sites. Hence, the small scale epidemics in this area can not solely be attributed to one batch of infected smolt distributed to all the four sites.

The basic assumption underlying the use of genotyping in tracing pathogen sources is that genetic similarity reflects kinship. Since there is little genetic variation in the ISAV genome, including the HE gene, and since the evolution of the virus probably does not conform to a molecular clock (Nylund *et al*., 2007), direct links between outbreak strains can not easily be drawn. The fact that viruses identical in the HE gene were identified on two different locations years apart shows that an identical sequence does not imply direct recent transmission events (Devold *et al*., 2006). However, relatively large genetic differences, such as those between the genogroups used in the present study, are suited to indicate that findings may not be compatible with direct transmission (Hungnes *et al*., 2000). Hence, this study presents a strong case for not rejecting the hypothesis that ISAV spreads locally and horizontally, even though there were few outbreak cases that were matched for risk factors and compared for genogroups. The study also supports the conclusions from Scheel *et al*. (2007) who attributed a significant proportion of ISA outbreaks to horizontal transmission from adjacent sites or sites within contact networks.

The HPR-region varies significantly between related isolates probably due to serial deletions. However, in this study in one occasion two isolates from the same outbreak belonging to G2 (200510A) and G3 (200510B) shared the same HPR (Figure 4, Table 2). This HPR type has mostly been found in G2 isolates, and strongly differs from the HPR sequences from the G3 isolates otherwise most related with 200510 B, suggesting a possible recombination event between 200510 A and B in the HPR. This, in addition to the fact that HPR appears to vary in the course of a single outbreak, confirms that HPR is not suited as an indicator of kinship between isolates.

The present study failed to find any significant patterns of genogroups related to smolt suppliers or broodfish companies. This may be due to the data being too fragmented and the number of outbreak sites too few. Furthermore, the design of the retrospective analysis regarding infected smolt or broodfish companies as risk factors may be flawed due to the complex infrastructure of the industry. These finding are in contrast to Nylund *et al*. (2007), who concluded that sources of eggs, i.e. the broodfish companies, best explained the origin of ISAV. These authors further suggested that some sort of transgenerational transmission may occur. No coherent analysis, however, was presented to support this hypothesis.

However, it is noteworthy that no outbreaks of ISA were reported in the freshwater phase of the salmon production cycle during 2003-2005, and only three outbreaks have been recorded in freshwater in Norway since 1984 (pers. com. Knut Falk). To study the importance of smolt or broodfish as risk factors there is a need for prospective studies with genotyping of ISAV in eggs and juvenile fish, followed by studies of outbreaks in sea sites coupled to ISAV positive eggs or juvenile fish.

The ISA outbreaks were distributed along most of the Norwegian coast and show a variable clinical picture. ISA seems to develop slowly which indicates that a site may be infectious for many months before a diagnosis is established. The present findings are thus in accordance with previous descriptions of ISA outbreaks in Norway (Thorud and Djupvik, 1988; Thorud and Håstein, 2003).

Numerous well boat visits and long transport distances (up to 1800 km) were characteristic for the ISA outbreaks. As we do not have information from non-diseased sites, the importance of such risk factors can not be evaluated. However, from Scotland it has been shown that numerous well boat visits were associated with ISA outbreaks (Murray *et al*., 2002). Since well boats are involved in many different operations, and ISA may stay non-detected for prolonged periods, the potentially concealed risk represented by well boats needs to be clarified.

ISA is controlled through different bio security measures at the production and transport levels. These measures focused initially on horizontal transmission. Our findings indicate that different forms of horizontal transmission may still be important for disease mitigation and underlines the necessity to maintain a strict biosecurity regime.

In conclusion, genotyping of virus isolates from ISA outbreaks supports associations between adjacent outbreaks. This is consistent with a process involving horizontal transmission of ISAV. The present study failed to find any significant patterns of genogroups related to smolt suppliers or brood fish companies.

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Appendix I

Epidemiological investigation of outbreaks of Infectious Salmon Anaemia (ISA) in 2005 - Case characteristics

In 2005 a total of 11 outbreaks of ISA were reported by the Norwegian Food Safety Authority. Four of the outbreaks occurred in the county of Finnmark, whereas the county of Møre and Romsdal, Trøndelag and Rogaland saw two outbreaks each. There was only a single outbreak in the county of Hordaland. All sea sites were breeding Atlantic salmon (*Salmo salar* L.) except one cage in one site with rainbow trout. However, ISA symptoms were not reported on the rainbow trout (Table 5). Six of the sites were reported as coast site, and four of the sites as fiord sites. Six of the outbreaks were identified in sea sites with polar circle, and four of the outbreaks in sea site with metal cages.

Six of the ISA outbreak cases reported that the basis for the ISA suspicion were due to reasonable evidence of the presence of ISA-virus in tissue or tissue material from two independent laboratory tests (IFAT and RT-PCR) (four outbreaks) , and/or post mortem findings consistent with ISA according to the OIE-Manual (four outbreaks). Three of the outbreaks sites in 2005 reported about contact/ epidemiological links to sites with confirmed ISA which lead to the suspicion of ISA. Isolation and identification of ISA-virus in cell culture from a single sample from any fish on farm was reported from one of the outbreaks. Information about the basis for the ISA diagnosis was missing on two of the questionnaires.

Dates for the suspicion of ISA outbreaks in 2005 were distributed from March until September. Nine of the outbreaks in 2005 were suspected within the first 6 month of 2005 (Table 5).

Two the outbreak sites reported that the site was included in an observation zone due to ISA outbreak. Two other sites reported that the sea site was included in an observation zone due to ISA outbreak and had also been included in an observation zone due to a previous ISA outbreak. One of the outbreak sites reported that the site had only been included in an observation zone due to a previous ISA outbreak in 1997. None of the ISA outbreaks sites in 2005 reported that ISA had earlier been diagnosed at the same sea site.

The mean weight of the fish in the different sea cages ranged from minimum 0.8 kg to maximum 6.4 kg with a mean weight of 3.6 kg by the time of sampling due to ISA suspicion. At the same time nine of the outbreak sites reported clinical symptoms consistent with ISA from at least on cage (Table 5).

The majority of the ISA outbreak sites reported other diseases than ISA causing increased mortality in the same production period. The most frequented reported disease was IPN, thereafter heart and skeletal muscle inflammation (HMSI), cardiomyopathy syndrome (CMS), winter ulcer, gill disease, epithyeliocystis and parvicapsula. Pancreas disease (PD) was not reported from any of the ISA outbreak sites (Table 5).

During the last three month before ISA was suspected the following stress of the fish were reported: Five of the sites reported grading or movement of fish. One of the sites reported washing of net pens, another one reported bad weather/storm (Table 5). Nine of the sites reported treatment for sea lice, and three of the sites reported slaughtering of fish from the sea site.

Information about daily temperature prior the ISA-suspicion was received for seven of the outbreak sites. Sites with ISA outbreaks in early spring reported sea temperature between 4^0C and 5^0C the past three month before ISA suspicion. The sites with ISA outbreaks during spring reported increasing average temperature from 4^0C to 11^0C. The site with ISA suspicion in late September 2005 reported decreasing sea temperature from approximately 12^0C down to 11^0C.

Smolts were mainly transferred to sea site during spring/summer time. The majority of smolts were age 1 when transferred to sea site. The mean number of smolt suppliers in 2005 for each sea site was two, ranging from one (min) to three (max). The mean number of smolts transferred to the different sea sites was 595,536 (min 425,300, max 776,400). On average the fish was kept in sea for 13 month (min 9, max 21,5) before ISA suspicion (Table 5).

Mortality numbers were reported for eight of the eleven ISA outbreaks. The information about mortality in percent per month the past three month before ISA was suspected showed that the mortality rates were variable. For 2005, maximum cage mortality on an ISA outbreak site varied from 0,5% to 23% the last month prior to the outbreak. Increased mortality was most often registered in only one or two cages on a site. For seven of the eight sites the mortality was increased in at least one cage in the month prior to the ISA suspicion (Table 5).

None of the sea sites with outbreaks of ISA was reported to be established before 1994. A fallow period before smolt was transferred to the sea site was reported from eight of the outbreak sites. For two of the sites information about fallowing was not reported. One of the ISA outbreak sites were used for aquaculture production for the first time. Only three of the sites reported to have an onshore base.

All sites reported visits by well boat (Table 5). In addition to well boats visit due to transferring smolt to the sea site, a number of other boat visits was reported, e.g. service boats (a single visit or regular), feeding boats (daily/weekly visits for some of the sites) , and well boats collecting fish for slaughter.

Seven of the sites reported diving activity due to inspection purposes at the sea site. Five of the sites reported joint operations with neighbouring sea sites, where three of these outbreak sites reported joint operations with sites with verified ISA outbreak in 2004 and 2005. Shared equipment for these joint operations was reported to be boats, net washer and tank for dead fish etc.

Two of the sites reported movement of fish to another sea site before ISA suspicion. None of the sites reported any escape of fish from the sea site.

The distance to the nearest sea route with well boat transport of salmon varied from 50 metres up to 15 kilometres. However, the majority of the sites reported about a distance below five km to the nearest transport route.

All of the ISA outbreak sites reported neighbouring sea sites within 10 km except one sea sites where the distance to neighbouring sea sites were more than 36 km.

Table 5. Summary of epidemiological reports on ISA outbreaks in 2005

Outbreak number	01/2005	02/2005	03/2005	04/2005	05/2005	06/2006
County	Møre og Romsdal	Finnmark	Finnmark	Finnmark	Møre og Romsdal	Sør-Trøndelag
Sea site ID	10214, Lid	10836, Vegglandet	10616, Vinnalandet	10834, Vinnaeidet	17495, Vullum	12407, Flesa
Species	Atlantic salmon[1]	Atlantic salmon[1]	Atlantic salmon[1]	Atlantic salmon[1]	Atlantic salmon[1] and Rainbow trout	Atlantic salmon[1]
Date of transfer to sea site	Des 2003 – May 2004	May 2003 – Jun 2003	May 2004 – Jun 2004	May 2004 – Jun 2004	Not reported	Apr 2004 – Aug 2004
Number of smolts transferred to sea site	539,500	Not reported	567,000	772,000	Not reported	425,300
Well boat visits[2]	12	7	5	6	Not reported	3
ISA suspected	3.03.2005	30.03.2005	19.04.2005	19.04.2005	27.04.2005	27.04.2005
Time period[3]	12,5	21,5	10,5	10,5	Not reported	10
Mortality rate[4]	0,01 - 0,8%	O,02 - 22,8%	0,1 – 1,2%	0,1 – 6,2%	Not reported	0,1 – 15,3%
Clinical signs	Diffuse, non specific symptoms, Anaemia	Symptoms consistent with ISA	Symptoms consistent with ISA	Symptoms consistent with ISA	Not reported	Symptoms consistent with ISA[5]
Stress	Nov 2004 - Dec 2004: Grading and movement of fish on the sea site	No stress of the fish were reported	No stress of the fish were reported	No stress of the fish were reported	Not reported	Mar 2005: Movement of fish on the sea site Mar 2005: Bad weather
Diseases other than ISA	Oct 2004: HSMI, Gill disease	Dec 2004: CMS Jul 2005: IPN	No other diseases reported	No other diseases reported	Not reported	Jul 2004: IPN
Contact with other ISA outbreak sites	No reported contact	Shared management, equipment and service boats with ISA outbreak 15/2004	Shared management, equipment and service boats with ISA outbreak 04/2005	Shared management, equipment and service boats with ISA outbreak 03/2005	No reported contact	No reported contact

Cont. Table 5

Outbreak no	07/2005	08/2005	09/2005	10/2005	11/2005
County	Hordaland	Rogaland	Rogaland	Finnmark	Nord-Trøndelag
Sea site ID	22095, Hågårdsneset	11925, Vintraviki	10113, Kobbavika	13143, Bondejorda	14245, Jakobsteinvik
Species	Atlantic salmon[1]	Atlantic salmon[1]	Atlantic salmon[1]	Atlantic salmon[1]	Atlantic salmon[1]
Date of transfer to sea site	Apr 2004- May 2004	Aug 2004 – Oct 2004	Apr 2004 – May 2004	Not reported	Apr 2004 – Jun 2004
Number of smolts transferred to sea site	585,542	776,400	642,908	Not reported	455,641
Well boat visits[2]	6	4	18	Not reported	9
ISA suspected	26.05.2005	3.06.2005	7.06.2005	25.08.2005	30.09.2005
Time period[3]	12,5	9	13,5	Not reported	16
Mortality rate[4]	Not reported	0,1 – 7,6%	0,02 – 2,4%	Not reported	0,0 – 0,5%
Clinical signs	Symptoms consistent with ISA	Symptoms consistent with ISA	Symptoms consistent with ISA	Symptoms consistent with ISA	Symptoms consistent with ISA
Stress	No stress of the fish were reported	Feb 2005: Movement of fish within the sea site	Apr 2005: Movement of fish within the sea site	Not reported	Jul 2005 – Sep 2005: Grading, movement of fish within the sea site, and cleaning
Diseases other than ISA	Jun 2004: IPN Mars 2005: Winter Ulcer	Sep 2004: IPN May 2005: HMSI	Sep 2004 (Epiteliocystis)	Not reported	Aug 2004: IPN, HMSI, Parvicapsula, Flexibacter Jan 2005: CMS Feb 2005: HMSI
Contact with other ISA outbreak sites	No reported contact	No reported contact	No reported contact	No reported contact	No reported contact

1 Sea-water farmed Atlantic salmon (*Salmo salar* L.)
2 Number of visits includes only transfer of smolts/fish to sea site
3 Average time period from transfer to sea site until ISA suspicion
4 Min and max monthly mortality at cage level the last three months before ISA was suspected
5 ISA symptoms were only reported on Atlantic salmon

References

1. Anon. Contingency plan for control of ISA in Norway 2004. Norwegian Food Safety Authority. 2004. 4-1-2007.

2. Cipriano RC and Miller O. 2003. Infectious Salmon Anemia: The Current State of Our Knowledge. In: Miller O, Cipriano, RC, and tech.coords. (Eds.) International response to infectious salmon anemia: prevention, control, and eradication. proceedings of a symposium; 3-4 September 2002. pp. 1-8. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior; U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service, New Orleans, LA. Washington, DC.

3. Cunningham CO, Gregory A, Black J, Simpson I and Raynard RS. 2002. A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. Bull. Eur. Ass. Fish. Pathol. 22:366-374.

4. Devold M, Falk K, Dale B, Krossoy B, Biering E, Aspehaug V, Nilsen F, and Nylund A. 2001. Strain variation, based on the hemagglutinin gene, in Norwegian ISA virus isolates collected from 1987 to 2001: indications of recombination. Dis. of Aquat. Org. 47:119-128.

5. Devold M, Karlsen M and Nylund A. 2006. Sequence analysis of the fusion protein gene from infectious salmon anemia virus isolates: evidence of recombination and reassortment. J. Gen. Virol. 87:2031-2040.

6. Hungnes O, Jonassen TO, Jonassen CM and Grinde B. 2000. Molecular epidemiology of viral infections. How sequence information helps us understand the evolution and dissemination of viruses. APMIS. 108:81-97.

7. Jarp J and Karlsen E. 1997. Infectious salmon anaemia (ISA) risk factors in sea-cultured Atlantic salmon, *Salmo salar*. Dis. of Aquat. Org. 28:79-86.

8. Lyngstad TM, Brun E, Jansen PA, and Heier BT. 2005a. Sammendrag av epidemiologiske rapporter om utbrudd av ILA i Norge 2003. Veterinærinstituttet, Oslo.

9. Lyngstad TM, Brun E, Jansen PA, and Heier BT. 2005b. Sammendrag av epidemiologiske rapporter om utbrudd av ILA i Norge 2004. Veterinærinstituttet, Oslo.

10. McClure CA, Hammell KL and Dohoo IR. 2005. Risk factors for outbreaks of infectious salmon anemia in farmed Atlantic salmon, Salmo salar. Prev. Vet. Med. 72:263-280.

11. Mjaaland S, Hungnes O, Teig A, Dannevig BH, Thorud K and Rimstad E. 2002. Polymorphism in the infectious salmon anemia virus hemagglutinin gene: importance and possible implications for evolution and ecology of infectious salmon anemia disease. Virology. 20;304:379-391.

12. Murray AG, Smith RJ and Stagg RM. 2002. Shipping and the spread of infectious salmon anemia in Scottish aquaculture. Emerg. Infect. Dis. 8:1-5.

13. Nylund A, Devold M, Plarre H, Isdal E and Aarseth M. 2003. Emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe: a new hypothesis. Dis. Aquat. Organ. 56:11-24.

14. Nylund A, Plarre H, Karlsen M, Fridell F, Ottem KF, Bratland A and Saether PA. 2007. Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). Arch. Virol. 152:151-179.

15. Scheel I, Aldrin M, Frigessi A and Jansen PA. 2007. A stochastic model for infectious salmon anemia (ISA) in Atlantic salmon farming. J. R. Soc. Interface (submitted).

16. Thorud K and Djupvik HO. 1988. Infectious anaemia in Atlantic salmon (Salmo Salar L.). Bull. Eur. Assoc. Fish Pathol. 8:109-111.

17. Thorud K and Håstein T. 2003. Experiences with Regulatory Responses to Infectious Salmon Anemia in Norway. In: O. Miller, R. C. Cipriano, and techn.coords. (Eds.) International response to infectious salmon anemia: prevention, control, and eradication. Proceedings of a symposium; 3-4 September 2002. pp. 155-159. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior; U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service, New Orleans, LA. Washington, DC.

18. Vågsholm I, Djupvik HO, Willumsen FV and Tveit AM. 1994. Infectious salmon anaemia (ISA) epidemiology in Norway. Prev Vet Med 19:277-290.

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