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ORIGINAL ARTICLE

ISA virus in Chile: evidence of vertical transmission

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Abstract Infectious salmon anaemia virus (ISAV), genus *Isavirus* (family *Orthomyxoviridae*), is present in all large salmon (*Salmo salar*)-producing countries around the North Atlantic. The target species for this virus are members of the genus *Salmo*, but the virus may also replicate in other salmonids introduced to the North Atlantic (*Oncorhychus* spp.). Existing ISA virus isolates can be divided into two major genotypes, a North American (NA) and a European (EU) genotype, based on phylogenetic analysis of the genome. The EU genotype can be subdivided into several highly supported clades based on analysis of segments 5 (fusion protein gene) and 6 (hemagglutinin-esterase gene). In 1999 an ISA virus belonging to the NA genotype was isolated from Coho salmon in Chile, and in 2007 the first outbreaks of ISA in farmed Atlantic salmon was observed. Several salmon farms in Chile were affected by the disease in 2007, and even more farms in 2008. In this study, ISA virus has been isolated from salmon in a marine farm suffering an outbreak of the disease in 2008 and from smolts with no signs of ISA in a fresh water lake. Sequencing of the partial genome of these ISA viruses, followed by phylogenetic analysis including genome sequences from members of the NA and EU genotypes, showed that the Chilean ISA virus belongs to the EU genotype. The Chilean ISA virus groups in a clade with exclusively Norwegian ISA viruses, where one of these isolates was obtained from a Norwegian brood stock population. All salmonid species in the southern hemisphere have been introduced from Europe and North America. The absence of natural hosts for ISA viruses in Chile excludes the possibility of natural reservoirs in this country, and the close relationship between contemporary ISA virus strains from farmed Atlantic salmon in Chile and Norway suggest a recent transmission from Norway to Chile. Norway export large amounts of Atlantic salmon embryos every year to Chile; hence, the best explanation for the Norwegian ISA virus in Chile is transmission via these embryos, i.e. vertical or transgenerational transmission. This supports other studies showing that the ISA virus can be transmitted vertically.

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Introduction

Infectious salmon anaemia (ISA) virus is a member of the family *Orthomyxoviridae*, genus *Isavirus*. It has an eight-segment genome and is present in most countries that farm Atlantic salmon (*Salmo salar*): Norway, Scotland, Ireland, Faroe Islands, Canada, USA and recently Chile ([4, 8, 9, 12, 14, 20, 23]). Based on comparisons of the genomes of all known ISA viruses, they can be divided into two major genotypes, i.e. a North American (NA) and a European (EU) genotype [3, 5, 10]. The EU genotype can be further divided into several genogroups or clades based on phylogenetic analysis of segments 5 and 6 [7, 17]. These clades include isolates from Norway, Scotland, Faroe Islands, Nova Scotia (Canada) and Main (USA). Based on analysis of the hemagglutinin–esterase (HE) gene, the clade consisting of isolates from Nova Scotia and Main is genetically distant from the EU isolates and should probably be given status as the third genotype of ISA viruses [17]. However, analysis of the fusion (F) protein gene suggests a closer relationship to the EU isolates [7]. In a phylogenetic analysis of 92 EU ISA virus isolates, more than 13 clades

are recognized. Among these, three isolates from Scotland and four isolates from the Faeroe Islands constitute two distinct geographical clades, but none of the Norwegian clades reflect specific geographical locations [17]. It has been suggested that the geographical pattern of ISA virus isolates in Norway reflects the origin of the brood fish, i.e. that vertical transmission could play a major role in the spread of ISA virus in Norwegian salmon farming [17]. Vertical transmission of ISA virus has been shown in a study of Norwegian brood fish (NBF) [15]. The ISA virus is also easily transmitted between individuals (horizontal transmission) in tanks or net pens and also to a certain degree between salmon in different nets within a site [22]. However, there are no studies documenting transmission between farming sites.

The emergence of ISA in Chile and the official detection of ISA virus in 2007 is the second documentation of ISA virus and the first case of ISA in the southern hemisphere and in Chile [8, 9]. ISA virus identical to members of the NA genotype was first detected in Coho salmon in Chile in 1999 [9]. It should be remembered that species in the fish genera *Oncorhynchus* and *Salmo* do not occur naturally in the southern hemisphere and nor should specific pathogens for these genera. Members of these two genera have been introduced into Chilean aquaculture several times, and every year the salmon farming industry in Chile imports embryos of Atlantic salmon from Norway and other countries. Hence, there is a continuous risk of importing pathogens that can be vertically transmitted or transmitted via infected embryos. The present study presents the partial genome sequences of ISA viruses collected from salmon smolts in a freshwater lake and from postsmolts at a sea site where an official outbreak of ISA took place in Chile. Segments 2, 5 and 6 were used in a phylogenetic analysis of these Chilean ISA viruses, comparing them to known ISA viruses from the Northern hemisphere.

Materials and methods

ISA virus

The present study is based on sequences of segments 2, (PB1, RNA-dependent RNA polymerase gene), 5 (F, the fusion protein gene), and 6 (HE, the hemagglutinin–esterase gene) from an ISA virus isolated from Atlantic salmon in sea water (isolate CH01/08), and on segment 6 from an ISA virus collected in fresh water (isolate CH02/08) in Chile in 2008 (Table 1). The seawater isolate came from a farm experiencing an official outbreak of ISA, while the freshwater isolate came from a smolt production site in a freshwater lake. The smolts did not show any signs of ISA. The salmon in both seawater and fresh water had their origin as eggs in Norway. The gene sequences from these isolates are compared to those of the corresponding genes from ISA virus isolates from Canada, Faeroe Islands, Norway, and Scotland (Table 1) (cf. [7, 16, 17]).

RT-PCR and sequencing

The ISA virus isolates were propagated in Atlantic salmon kidney cells (ASK cells), and RNA was extracted using Trizol reagent (Life Technologies) according to standard protocols [6]. Reverse transcription, PCR and sequencing were performed as described by Nylund et al. [17]. The primers used to obtain the different gene sequences are presented in Table 2. The PCR products were stored at 4°C before sequencing, and the primers used for PCR were also used for sequencing.

Phylogeny

Sequence data were assembled with the help of Vector NTI software (InforMax, Inc.), and GenBank searches were done with BLAST (2.0). The Vector NTI Suite software package was used for multiple alignments of nucleotide sequences. To perform pairwise comparisons between the different sequences from the ISA virus isolates, the multiple sequence alignment editor GeneDoc was used. Sequences already available in the EMBL nucleotide database were also included in the comparisons (cf. [7, 17]). Phylogenetic trees were obtained by analysis of the PB1, F and HE protein genes (Table 1). Trees were constructed using TREE-PUZZLE 5.2 (Available at: http://www.tree-puzzle.de) with maximum likelihood as the optimality criterion and eight-category gamma distribution to describe substitution-rate heterogeneities (cf. [7, 17]). Phylogenetic trees were drawn using TreeView [18].

Results

The sequences of segments 2, 5 and 6 from the seawater ISA virus isolates from Chile were determined (accession nos: EU851041, EU851042, EU851043), and segment 6 from this isolate (CH01/08) is identical to segment 6 from the freshwater isolate (CH02/08). The other segments from CH02/08 were not sequenced. Phylogenetic analysis of all three segments showed that the Chilean ISA virus isolates belong to the EU subtype (Figs. 1, 2, 3). Based on analysis of segment 2, the closest relative is an ISA virus isolate from a marine salmon farm in central Norway, while ISA virus isolates from NBF are among the closest isolates in the phylogenies based on segments 5 and 6. The clades containing the Chilean ISA virus, in all three phylogenies,

Table 1 An overview of ISAV isolates and the accession numbers of gene segments 2 (PB1 gene), 5 (F gene) and 6 (HE gene)

Locality	Year	Code	PB1 gene Segment 2 Accession no	HE gene Segment 6 Accession no	F gene Segment 5 Accession no
Faeroe Islands					
Færøyene	2002	F72b/02	–	AY971656	AY853918
Færøyene	2002	F72/02	–	AF536263	AY853917
Scotland					
Loch Nevis	1998	Scot43/98	AF262392	AF302803	AY853947
390/98	1998	ISA98/01	–	ISA276859	ISA277461
Scotland	1998	–	AJ242808		
Canada					
Bay of Fundy, New Brunswick	1997	–	AF262399	–	–
		–	AF404346	–	–
		–	DQ520595	–	–
		–	DQ520596	–	–
Chile					
Chile, marine site	2008	CH01/08	EU851041	EU851043	EU851042
Chile, freshwater lake	2008	CH02/08	–	EU851043	–
Norway					
Hordaland					
Eikelandsosen	1987	H1/87	AF262394	–	–
Golten	1989	H2/89	AF262398	–	–
Sotra	1992	H7/92	AJ002475	–	–
Varaldsøy	1996	H17/96	AF262397	–	–
Bømlo (Bremnes)	1998	H36/98	AF262391	AF302799	AY853958
Strandebarm	1998	H40/98	–	AF364877	AY853959
Øygarden	2000	H51/00	–	AF364882	AY853931
Sørnes	2000	H56/00	–	AF364880	AY853946
Sogn og Fjordane					
Selje	1995	SF14/95	DQ785198	–	–
Gulen	1998	SF41/98	–	AF364871	AY853961
Brekke	1998	–	AF262390	–	–
Fjaler	2000	SF57/00	–	AF364890	AY853939
Leiholmane (Gulen)	2002	SF70/02	–	AY127880	AY853938
Brunsvik	2002	SF71/02	–	AY127881	AY853936
Brekke	2004	SF83/04	AY744389	–	–
Møre og Romsdal					
Lepsøy	2000	MR52/00	–	AF364892	AY853949
Ørskog	2001	MR60/01	–	AY127876	AY853944
Smøla	2001	MR61/01	–	AY127877	AY853935
Herøy	2001	MR62/01	–	AY127878	AY853937
Sør Trønderlag					
Hitra	1996	ST21/96	DQ785189	AF364886	AY853952
Frøya	1997	ST25/97	–	AF364885	AY853926
Frøya	1997	ST27/97	–	AF364897	AY853929
Åfjord	1997	ST28/97	–	AF364875	AY853927
Hitra	1999	ST44/99	AF262395	AF302803	AY853954
Nord Trønderlag					
Storbrannholmen	2003	NT81/03	–	AY973184	AY853955

Table 1 continued

Locality	Year	Code	PB1 gene Segment 2 Accession no	HE gene Segment 6 Accession no	F gene Segment 5 Accession no
Nordland					
Meløy	1989	N5/89	DQ785191	–	–
Vestvågøy	1993	N9/93	DQ785197	–	–
Svolvær	1996	–	AF262393	–	–
Torgnes	1997	N29/97	–	AF364872	AY853920
Dønna	1998	N32/98	–	AF364883	AY853921
Troms					
Gullesfjord	1993	T10/93	AF262396	AF302801	AY853922
Senja	1996	T22/96	DQ785195	AF364889	AY853968
Rotsundet	2002	T73/02	–	AY971663	AY853923
Uløy, Rotsundet	2003	T74/03	–	AY971664	AY853924
Finnmark					
Lille Torskefjord	2004	FM86/04	EU851045	AY971659	AY853943
Vir28(94/09/579)	1994		DQ785201	–	–
Unkown location					
Norwegian brood fish	2005	NBF	–	DQ108607	EU851044
SK779/06	2006	–	EU118816	–	–

The names of the different localities are given in the first column. The code includes the number given to the isolates and the year of collection. Isolates from North America are not included in the phylogenies based on segments 5 and 6

Table 2 Primers used for PCR and sequencing of segments 2 (PB1 gene), 5 (F gene) and 6 (HE gene) from the ISA virus isolates from Chile

Segment	Name	Sequence (5′–3′)	Reference
2	PB1-1F	AGCAAAGAACGCTCTTTAATAACCATG	Krossøy et al. [10, 11]
2	PB1-4F	CAACAGGTTTCAGAGGAAGAACCA	Krossøy et al. [10, 11]
2	PB1-6F	CAGGTCTACTGTTGTAGTGAAGGC	Krossøy et al. [10, 11]
2	PB1-9F	GGGAACAGAAAATACCAAGAACTGAAGA	Krossøy et al. [10, 11]
2	PB1-5R	TCCATGTTCCATCCACTCTACTCC	Krossøy et al. [10, 11]
2	PB1-9R	TCGTCAATTCCGTTATTACACACA	Krossøy et al. [10, 11]
5	S5-F1	AGTTAAAGATGGCTTTTCTAACAATT	Devold et al. [7]
5	S5-F10	ACCAAACAAAAGTTAAAGATGG	Present study
5	S5-R3	TTCTAAATTATCCAATAAAGGTCCTG	Devold et al. [7]
5	S5-R10	CAAAATATAAGTTATGTACAG	Present study
6	S6F1	GCAAAGATGGCACGATTC	Devold et al. [5]
6	HansF3	CATCCCAACTTCGATGACACTGG	Present study
6	HansR3	TCCCAAAACCTGCTACACCC	Present study
6	9ZR6	CATAGTTGTCTTTCTTTCATAATC	Present study

include only Norwegian ISA virus isolates as the other members. The clades containing the Chilean ISA viruses were all highly supported.

The partial sequence of segment 2 [2,001 nucleotides, position 127–2128 in the open reading frame (ORF)] of the Chilean ISA viruses differs by only one nucleotide from that of the most closely related Norwegian isolate, ST21/96 (accession no: DQ785189), but they have identical deduced amino acid (aa) sequences. The Norwegian ISA virus isolates included in the analysis of segment 2 differ at 78 (3.9%) nucleotide positions (comparing 2,001 nt in the ORF) in the PB1 gene sequence, resulting in 18 putative aa substitutions (2.7%). These variable positions in the segment 2 sequences are evenly distributed throughout the 2,001 nt in the alignment, and a large number of these are neutral substitutions.

The Chilean ISA virus isolates had an insert in segment 5 (F protein gene) preceding nucleotide 794 in the ORF of

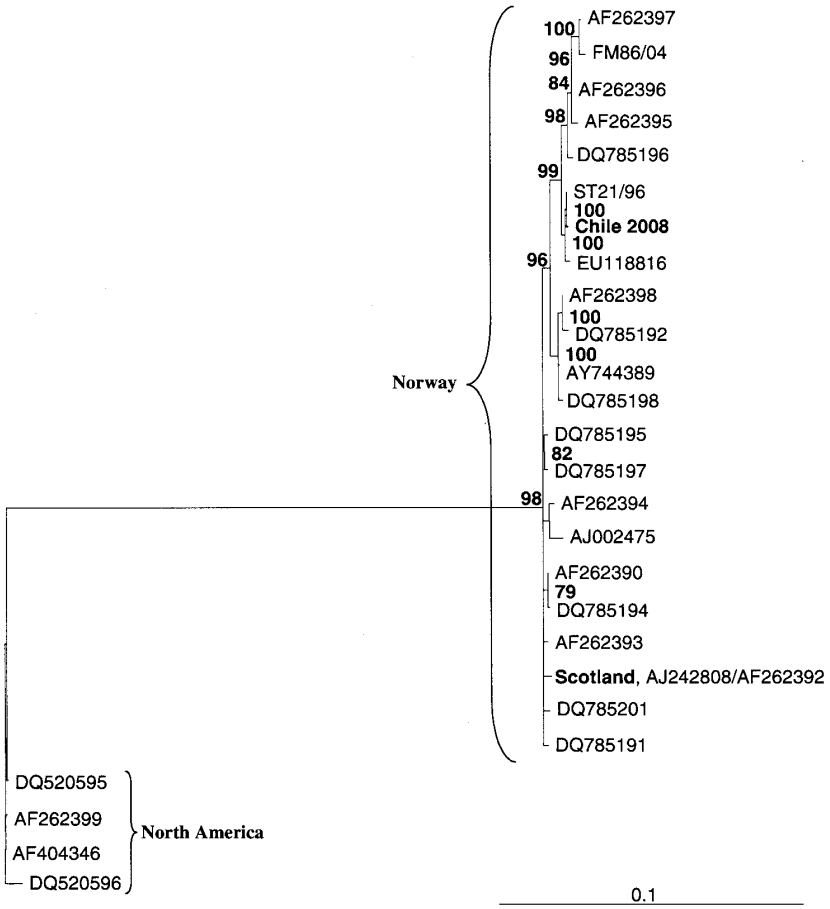

Fig. 1 Phylogenetic tree showing the relationship between ISAV isolates from Europe, Chile and North America based on 2,001 nucleotides (nucleotide 127–2128 in the ORF) encoding the PB1 protein. Phylogram resulting from maximum-likelihood analysis in TREE_PUZZLE. The best-fitting nucleotide substitution model was used during maximum likelihood analysis. The maximum-likelihood trees were bootstrapped (25,000 quartet puzzling steps) in TREE_PUZZLE. The *scale bar* shows the number of nucleotide substitutions as a proportion of branch lengths

this gene. The insert was identified by using a Blast search. The 33-nucleotide-long insert is identical to a sequence from segment 2 (PB1 gene), including positions 397–429 in the ORF of the PB1. The position of the insert in segment 5 is the same as that previously seen in a selection of Norwegian ISA virus isolates, i.e. in front of a putative cleavage site for trypsin, aa R^{267} (cf. [7]). The predicted aa sequence of the insert in segment 5 has the following sequence: KGKSANDIISD. The nucleotide sequences (1,290 nucleotides, position 39–1329 in the ORF) from segment 5 of the Chilean and the NBF ISA virus isolates differ at eight nucleotides. Excluding the insert in the Chilean isolate, these two isolates differ at only two aa positions. The alignment of the Norwegian ISA virus isolates included in this study show variation at 70 (5.4%) nucleotide positions (comparing 1,290 nt in the ORF) in the F gene sequence, resulting in 26 (6.0%) aa substitutions, excluding the inserts in the isolates; MR60/01, MR61/01, MR62/01, SF57/00, SF70/02, and SF72/02.

In the analysis of the HE gene, the Chilean ISA virus isolates form a clade with the same four Norwegian isolates as in the phylogeny based on the F protein gene. The Chilean isolates differ from the NBF isolate at four nucleotide positions (based on 952 nt, position 63–1015 in the ORF), resulting in three putative aa substitutions, while they differ in three nucleotide position compared to ISA virus isolate ST25/97, resulting in two putative aa substitutions. The alignment of the Norwegian ISA virus isolates included in this study show variation at 48 (5.0%) nucleotide positions (comparing 952 nt in the ORF) in the HE gene sequence, resulting in 18 (5.7%) putative aa substitutions.

Discussion

The distribution of ISA virus reflects the natural pattern of occurrence of members of the genus *Salmo* (*S. Salar* and *S.*

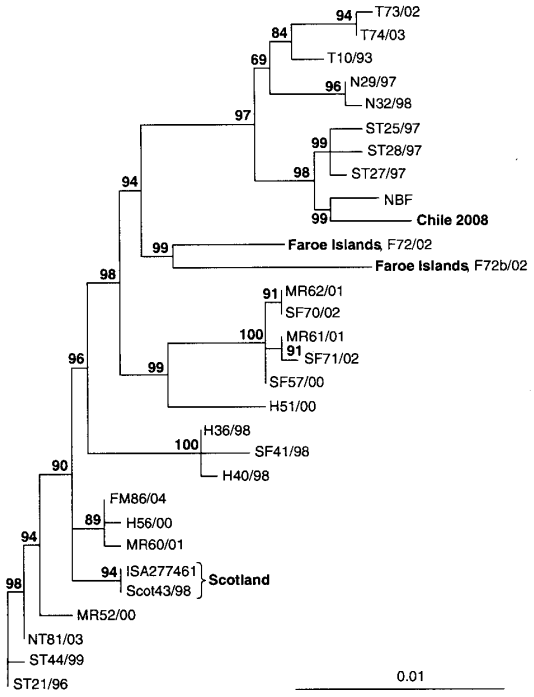

Fig. 2 Phylogenetic tree showing the relationship between ISAV isolates from Europe and Chile based on 1,290 nucleotides (nucleotide 39–1329 in the ORF) encoding the F protein. Phylogram resulting from maximum-likelihood analysis in TREE_PUZZLE. The best-fitting nucleotide substitution model was used during maximum-likelihood analysis. The maximum-likelihood trees were bootstrapped (25,000 quartet puzzling steps) in TREE_PUZZLE. The *scale bar* shows the number of nucleotide substitutions as a proportion of branch lengths

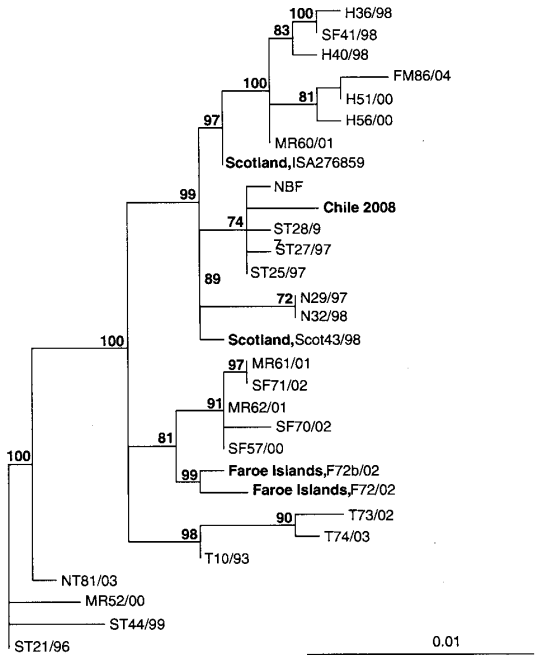

Fig. 3 Phylogenetic tree showing the relationship between ISAV isolates from Europe and Chile based on 952 nucleotides (nucleotide 63–1015 in the ORF) encoding the HE protein. Phylogram resulting from maximum-likelihood analysis in TREE_PUZZLE. The best-fitting nucleotide substitution model was used during maximum-likelihood analysis. The maximum-likelihood trees were bootstrapped (25,000 quartet puzzling steps) in TREE_PUZZLE. The *scale bar* shows the number of nucleotide substitutions as a proportion of branch lengths

trutta), i.e. presence on the East coast of North America (Canada and USA) and north Western Europe (Norway, Scotland, Ireland, and the Faroe Islands). The virus has not been found in *Oncorhynchus* species on the west coast of North America. The reasons for this pattern are, of course, that the ISA virus is a *Salmo* virus and that these countries have a salmon farming industry, which has made a naturally occurring virus in these areas visible to the industry and researchers. There is also a salmon farming industry in the southern hemisphere, but in those areas (Tasmania and Chile), there are no natural populations of salmonids, and hence no natural occurrence of *Salmo* viruses. However, salmonids have been introduced to Australia and Chile as fertilized eggs (embryos), i.e. introduced into natural water systems and farming sites. Movement of biological material always involves a chance of moving unwanted organisms like parasites and bacteria, but also viruses. It has already been documented that the ISA virus may be vertically or transgenerationally transmitted [15], and it has been suggested that vertical transmission plays a major role in the spread of ISA virus in the Norwegian salmon farming industry [17]. The latter suggestion is based on a combination of screening data showing that ISA virus has a high prevalence in Norwegian smolt farms and that genotyping of ISA virus from marine production sites shows a pattern consistent with a high frequency of vertical transmission [17]. It seems as if different NBF populations are carrying specific ISA viruses reflecting the brood stock population and generation. Based on these data, it has been expected that the ISA virus and the disease (ISA) should emerge in Chile, since a large number of Atlantic salmon embryos are imported every year to supply the Chilean aquaculture industry. The first ISA virus in Chile was detected as early as 1999 in Coho salmon [9]. This virus was of the NA genotype and could only have been introduced to Chile via transport of embryos from North America to Chile. The sequence identity of the HE gene of this ISA virus isolate was identical to HE gene sequences of contemporary Canadian ISA viruses, suggesting that the

introduction to Chile must have occurred shortly before the detection of the virus [9].

The ISA virus isolates, CH01/08 and CH02/08, were isolated from Atlantic salmon suffering from ISA at a marine production site, and from smolt at a production site in a freshwater lake in Chile. They are nearly identical genetically to contemporary Norwegian isolates, including an isolate (NBF) from a Norwegian Atlantic salmon brood stock population. The most closely related Norwegian ISA virus isolates were collected in the period from 1997 to 2005. The brood stock population belongs to a Norwegian brood stock company exporting large numbers of Atlantic salmon embryos to Chile. Based on the following facts, (a) that the Chilean ISA virus isolates group phylogenetically with contemporary Norwegian genotypes, (b) the existence of a large export of Atlantic salmon embryos from Norway to Chile, (c) the absence of natural populations of salmonids in Chile (all salmonids species were introduced to the Chilean fauna), and (d) documentation that ISA viruses can be transmitted vertically, it can be safely concluded the ISA virus isolates, CH01/08 and CH02/08, must have been recently (possibly during the last 10 years) transmitted via infected Atlantic salmon embryos from Norway to Chile (cf. [17]). The ISA virus isolates in Chile give solid evidence of vertical or transgenerational transmission of ISA virus.

It has been claimed that Chilean ISA virus isolates have a unique genetic feature that is not detected in isolates from any other salmon-producing countries [8]. This unique feature is an insert of 33 nucleotides in front of a putative trypsin-cleavage site (R^{267}); i.e. the cleavage site is one of two possible sites for proteolytic cleavage of the precursor fusion protein F_0 [1, 7]. The insert is identical to a sequence stretch of 33 nucleotides in the ORF of segment 2 (PB1 gene) (accession no: EU851041). However, an insert in this region of the F protein gene is not a unique genetic signature of the Chilean isolates, since such inserts have been found in this gene segment of several Norwegian isolates [7, 13]. These inserts can be derived from several different parts of the ISA virus genome and are a result of recombination events ([7], present study). The importance or significance of these inserts is not known, but it has been speculated that they may influence the accessibility of the cleavage sites to trypsin by perturbing the structure of F_0 [7]. It has been shown that a 30-nucleotide insert in front of the HA_0-cleavage site in influenza A virus from chickens may change the virus from a low-pathogenic virus (LPAI) to a highly pathogenic virus (HPAI) [21]. Phylogenetic analysis of HPAI viruses shows that they do not represent separate phylogenetic lineages but appear to be derived from low-pathogenic strains ([2, 19]). Hence, if anything, the insert in the F protein gene of the Chilean ISA virus isolates suggests a Norwegian origin, since such inserts have, so far, only been found in three different clades of Norwegian ISA viruses [7].

The detection of a Norwegian ISA virus genotype in Chile shows that the Chilean aquaculture industry has to restructure the production of Atlantic salmon to reduce the risk of introducing exotic diseases. A better control or reduction of import of embryos from Europe and North America, areas with natural populations of salmonids, should be considered to reduce the risk of importing salmon-specific pathogens that are not naturally present in Chile. The Chilean and Norwegian salmon industries, should also aim at producing ISA-virus-free brood stock in controlled environments. This can be achieved by screening brood fish for the presence of asymptomatic carriers of ISA virus, and hence avoid circulation of this pathogen in the production cycle. To prevent possible horizontal transmission of ISA virus in the Chilean salmon industry it should consider (a) avoiding production of smolt in lakes with strong populations of salmonids susceptible to ISA virus, (b) keeping only one generation at each marine site, and (c) introducing a minimum distance between marine sites for production of Atlantic salmon in the sea. The exact distance has to be considered separately for each area, depending on currents and other environmental factors.

This study shows that the same pathogenic ISA virus isolates are present in both a freshwater and a seawater site for production of Atlantic salmon and that these isolates must have a Norwegian origin. However, to get a better understanding of the transmission routes for ISA virus within Chile, it will be necessary to genotype ISA virus from a majority of ISA outbreaks, acquire data about the origin of eggs (brood fish), smolt production sites, movement of smolt to sea sites, and possible connections between sea sites. Work to obtain such data is ongoing.

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