Arch Virol (2006) 151: 861–874
DOI 10.1007/s00705-005-0687-6

Archives of Virology
Printed in Austria

Genetic stability within the Norwegian subtype of salmonid alphavirus (family *Togaviridae*)

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Received July 25, 2005; accepted October 27, 2005
Published online December 15, 2005 © Springer-Verlag 2005

Summary. Salmonid alphavirus (SAV) (family *Togaviridae*) causes mortality in Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* W.) in Norway, France, UK, and Ireland. At least three subtypes of SAV exist: SPDV in UK/Ireland, SDV in France/UK, and the recently reported Norwegian salmonid alphavirus (NSAV) in western Norway. During 2003 and 2004, disease caused by NSAV was reported for the first time in northern Norway, more than 800 km away from the enzootic area in western Norway. The present study has investigated the phylogenetic relationships among 20 NSAV isolates, based on a 1221-nt-long segment covering part of the capsid gene, E3, and part of the E2 gene, collected over a period of eight years. The results revealed genetic homogeneity among NSAV isolates, including those from northern Norway. The SDV or SPDV subtypes were not found in diseased Norwegian fish. A substitution rate of 1.70 ($\pm$1.03) $\times$ 10^{-4} nt subst/site/year was obtained for the NSAV subtype by maximum likelihood analysis. The second aim of this study was to clarify whether NSAV changes genotypically in cell culture by culturing a NSAV isolate through 20 passages in CHSE-214 cells. Sequencing of almost the entire genome (11530 nt) after 20 passages revealed four nucleotide substitutions, all resulting in amino acid substitutions. One of these substitutions, serine to proline in E2 position 206, was also found to have occurred in field isolates.

Introduction

Pancreas disease (PD) and sleeping disease (SD) in farmed salmonid fish has been known since the 1976 outbreak of PD in farmed Atlantic salmon (*Salmo salar* L.) in Scotland [28] and several outbreaks of SD affecting French rainbow trout (*Oncorhynchus mykiss* W.) in the 80s and early 90s [2]. The first study of PD in farmed Atlantic salmon in Norway was published in 1989 [33]. During the last decade, the disease has become a growing problem for the major salmonid-

producing countries in Europe, being the number one cause of economic loss in Ireland [7]. The causative agents of PD and SD, salmon pancreas disease virus (SPDV/SAV-1) and sleeping disease virus (SDV/SAV-2), respectively, are two closely related members of the genus *Alphavirus* (family *Togaviridae*) [42, 52] that constitute two subtypes of salmonid alphavirus (SAV) [35, 51]. A third subtype of SAV from Norway, Norwegian salmonid alphavirus (NSAV/SAV-3) has recently been described, and it is likely that all previously reported cases of PD in Norway have been caused by this subtype [14]. The complete genome similarity among these subtypes is $>90\%$ on the nucleotide level [14, 51]. In Norway, PD has caused mortality and economic loss in Atlantic salmon and rainbow trout farms in a relatively restricted area of western Norway. However, during the years 2003 and 2004 there were several reports of the disease appearing in new areas of Norway, including the northernmost counties Nordland, Troms, and Finnmark. Since the disease caused by SPDV and NSAV has been reported from saltwater only, it has traditionally been believed that the natural reservoirs of these subtypes are marine, and that spreading occurs by horizontal transmission in the sea [18, 26]. The northern Norway incidents do, however, provoke further discussion on how transmission in the field may occur.

Experimental *in vivo* transmission of SAV has failed in several studies to yield the high mortality rate that is commonly observed in field outbreaks. Some of these studies have been based on cell-culture-adapted strains of SAV [3, 23, 25, 51], and this has caused speculations concerning the evolutionary stability of the virus *in vitro*. Although *in vitro* virus evolution is not directly comparable to evolution *in vivo*, combined knowledge on these two fields may be helpful when substitution candidates for further virulence studies are to be identified.

The major aim of this study was to get an overview of the genetic diversity within the NSAV subtype. Such information can hopefully be used for further studies within molecular epizootiology and provide insight to questions concerning routes of transmission in the aquaculture industry [15]. Based on previous studies of terrestrial alphaviruses [20, 27, 38, 39, 46, 49] and the sequence comparison by Hodneland et al. [14], a 1221-nt-long gene segment covering E3, parts of E2, and parts of the capsid (C) gene was chosen for this study. A total of 20 SAV isolates from Norway, covering a time period of eight years (1997–2004), were sequenced, and the phylogenetic relationship among them was calculated.

A secondary aim of this study was to clarify whether and, if so, how SAV changes genetically in cell culture. The NSAV isolate SAVH20/03 was cultured through 20 passages in Chinook salmon embryo (CHSE-214) cells, sequenced, and compared to a near full-length passage three sequence previously published by Hodneland et al. [14].

Materials and methods

Virus isolates

Tissues (heart, ventricle) were collected from Atlantic salmon and rainbow trout diagnosed with classical PD, with the exception of the isolates SAVH10/02 and SAVH20/03, which came

Table 1. Virus isolates used for sequence comparison studies

Isolate	Location	Time of sampling	Host	Diagnosis*	Accession no.
Northern Norway					
Finnmark					
SAVF29/03	Vest-Finnmark	December, 2003	*S. salar*	PD	DQ122127
Troms					
SAVT28/03	Nord-Troms	December, 2003	*S. salar*	PD	DQ122128
Nordland					
SAVN32/04	Vesterålen	September, 2004	*S. salar*	PD	DQ122129
Western Norway					
Sogn og Fjordane					
SAVSF21/03	Solund	February, 2003	*S. salar*	PD	DQ122130
SAVSF22/03	Solund	February, 2003	*S. salar*	PD	DQ122131
Hordaland					
SAVH30/04	Askøy	June, 2004	*S. salar*	PD	DQ122132
PD97.N2	Osterøy	May, 1997	*S. salar*	PD	DQ122133
PD97.N3	Osterøy	Autumn, 1997	*O. mykiss*	PD	DQ122134
SAVH02/99	Osterøy	May, 1999	*S. salar*	PD	DQ122135
SAVH20/03	Øygarden	April, 2003	*S. salar*	CMS	DQ122136
SAVH10/02	Øygarden	2002	*S. salar*	CMS	DQ122137
SAVH26/03	Fjell, Sotra	October, 2003	*S. salar*	PD	DQ122138
SAVH03/00	Austevoll	June, 2000	*S. salar*	PD	DQ122139
SAVH04/00	Austevoll	July, 2000	*S. salar*	PD	DQ122140
SAVH05/01	Nordhordland	November, 2001	*S. salar*	PD	DQ122141
SAVH25/03	Askøy	May, 2003	*S. salar*	PD	DQ122142
SAVH23/03	Askøy	April, 2003	*S. salar*	PD	DQ122143
SAVH24/03	Øygarden	April, 2003	*S. salar*	PD	DQ122144
Rogaland					
SAVR01/99	Ryfylke	April, 1999	*S. salar*	HSS	DQ122145
SAVR31/04	Haugaland	August, 2004	*S. salar*	PD	DQ122146

*Set by local veterinary service

from fish diagnosed with cardiomyopathy syndrome (CMS) [14], and the isolate SAVR01/99, which came from fish suffering from haemorrhagic smolt syndrome (HSS) [29]. The isolates included were collected from the Norwegian counties Finnmark, Troms, Nordland, Sogn og Fjordane, Hordaland, and Rogaland, during the years 1997–2004 (Table 1, Fig. 1).

RNA extraction and reverse transcriptase (RT) PCR

Total RNA was extracted from heart tissue or cell culture and transcribed into cDNA as described by Devold et al. [9]. The PCR was empirically optimized with regard to temperature (55–64 °C) and $MgCl_2$ concentration (1.0–3.0 mM) by running gradients on an

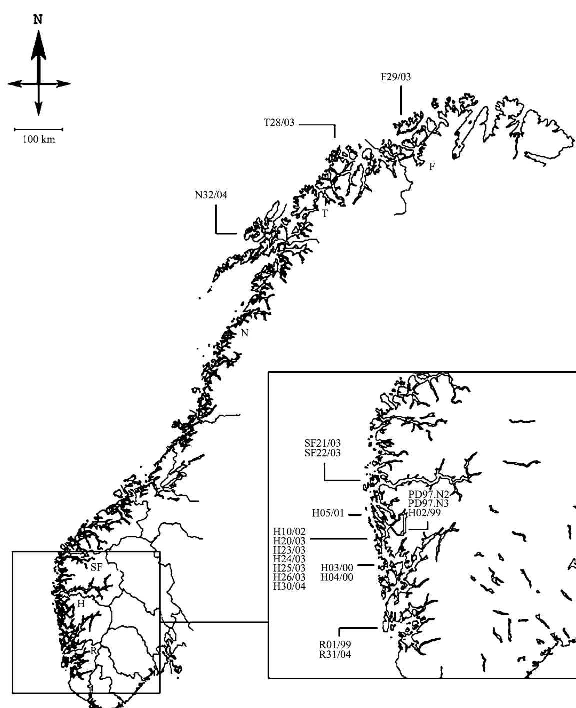

Fig. 1. The coastline of Norway. Locations where the NSAV isolates were collected are indicated. The isolate prefix (SAV-) is left out for convenience. Counties that are discussed in the text are indicated on the map as follows: F = Finnmark, T = Troms, N = Nordland, SF = Sogn og Fjordane, H = Hordaland, R = Rogaland

Table 2. Primers used for PCR and sequencing. Positions are presented as position according to the near full-length SAVH20/03 sequence (accession no. AY604235)

Primers	Sequence	Position
F1600	5′-CGGCACTATCAGAGTGGAGGA-3′	8377–8397
F2234	5′-CGGGTGAAACATCTCTGCG-3′	9015–9033
R2357	5′-AGGATGTAGTGGCCGGTGG-3′	9120–9138
SAV20R	5′-GGCATTGCTGTGGAAACC-3′	9746–9763

Eppendorf® Mastercycler Gradient. The overlapping primer combinations F1600/R2357 and F2234/SAV20R (Table 2) were used to cover a 1347-nt-long segment of the structural ORF. This segment included the last 214 nucleotides of the C gene, the entire E3 gene and the first 960 nucleotides of the E2 gene. The total reaction volume of 50 μl contained 5.0 μl 10× running buffer (Promega), 4.0 μl 2.5 mM dNTP, 3.0 μl 25 mM $MgCl_2$, 1.0 μl 10 mM forward primer, 1.0 μl 10 mM reverse primer, 0.3 μl Taq polymerase (Promega), 33.7 μl ddH_2O, and 2.0 μl cDNA template.

The reactions were run under the following conditions: Initial denaturation was done at 95 °C for 5 min. Then 40 cycles were carried out as follows: Denaturation at 94 °C for 30 sec, annealing at 59 °C for 45 sec, and elongation at 72 °C for 60 sec. Extension was done for 10 min at 72 °C. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen), following producer recommendations.

Sequencing and sequence analysis

An ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Perkin-Elmer) was used according to producer recommendations for sequencing the purified PCR product. In order to obtain the consensus sequence, the PCR

products were not on any occasion cloned prior to sequencing, thereby avoiding the possible bias represented by quasispecies and sequence errors introduced by the non-proofreading Taq-polymerase enzyme. The PCR primers F1600, R2357, F2234, and SAV20R, were used in the sequencing reaction, and all isolates were sequenced in both directions.

Sequences were processed (cropped to 1221 nt) and aligned by Vector NTI Suite version 9.0.0 (Informax) and GeneDoc (available at www.psc.edu/biomed/genedoc). Alignments including the SPDV (F93-125, accession no. AJ316244) and SDV (S49P, accession no. AJ316246) reference isolates were imported into PAUP v4.0 [41], and phylogenetic analysis using likelihood were conducted as follows: A) Modeltest v3.6 [34] was used to identify one of 56 models best fitting the dataset, based on the Akaike Information Criterion. B) A model with base frequencies (A = 0.2373, C = 0.3052, G = 0.2718, T = 0.1857), six substitution types with six-parameter instantaneous rate (A–C = 1.0000, A–G = 5.1694, A–T = 0.3855, C–G = 0.3855, C–T = 13.0220, G–T = 1.0000), and among-site rate variation with gamma shape value 0.4585 was employed. C) A maximum likelihood tree was obtained. The tree was bootstrapped using 1000 replicates and imported into and drawn in TreeView [32] with SDV and SPDV reference sequences as outgroups. D) The nucleotide substitution rate for the NSAV subtype (including both synonymous and non-synonymous substitutions) with confidence intervals (0.95) was calculated for the NSAV subtype (n = 20) by BASEML in the PAML v3.14 package [53], using the single rate dated tips (SRDT) model [36]. A molecular clock was tested by a likelihood ratio test, based on the likelihood score of the SRDT model tree and the likelihood score of a different rate (DR) model tree (no clock; branches are allowed to evolve with independent rates), also obtained with BASEML.

Cell culture

Chinook salmon embryo (CHSE-214) cells were cultured in 75 cm^2 Nunclon™ bottles as previously described [14]. Cells were initially infected with passage three of the isolate SAVH20/03 at a multiplicity of infection (moi) of ca. 0.0003 $TCID_{50}$. Infected cells were grown at 14 °C for 8–14 days before passaging (1:150 dilution). The supernatant was removed and RNA was extracted from the infected monolayer and transcribed into cDNA. cDNA from passage 20 was used for PCR and sequencing of the near full-length NSAV genome, nucleotide position 1 to 11530, according to the passage 3 sequence previously reported by Hodneland et al. (accession no. AY604235) [14]. The passage 20 sequence was aligned and compared to the sequence from passage three at the nucleotide and deduced amino acid levels.

Results

Phylogenetic studies

Eight nucleotide differences were registered among the 20 NSAV isolates (Table 3), setting the nucleotide diversity to approximately 0.66% for the investigated genome region. Two substitutions were found in the E3 gene, while the last six resided within the E2 gene. Two substitution sites were localized in relatively close proximity to each other, the E2 positions 611 and 616.

Several of the isolates were identical in the screened genome region, and the 20 isolates constituted nine different genetic variants. Some of these differed by only a single nucleotide substitution. When comparing amino acid sequences, the nine genetic variants are reduced to six groups based on amino acid differences (Table 3). The reduction is due to the isolates SAVH20/03 and SAVN32/04 residing in the EATRS group (name refers to amino acid identity of the five substitution

Table 3. Nucleotide and amino acid substitutions found in 20 NSAV isolates. The prefix (SAV-) is not included in isolate names for convenience. All positions are numbered according to the SAVH20/03 isolate (accession no. AY604235) and are presented as nucleotide/amino acid position within the respective genes. Amino acid positions and substitutions are printed in bold and groupings based on amino acid differences are referred to in the text as the amino acid identity of the five amino acid substitution sites

Isolates	E3 52	E3 115/**39**	E2 10/**4**	E2 66	E2 271/**91**	E2 561	E2 611/**204**	E2 616/**206**
H10/02, H23/03, H24/03, H25/03, H26/03, T28/03, F29/03, H30/04	A	G/**E**	G/**A**	C	A/**T**	C	G/**R**	T/**S**
PD97.N2, H03/00, H04/00, H05/01	A	A/**K**	G/**A**	C	A/**T**	T	G/**R**	T/**S**
SF21/03, SF22/03	A	A/**K**	A/**T**	C	A/**T**	T	A/**K**	C/**P**
PD97.N3	A	A/**K**	A/**T**	C	A/**T**	T	G/**R**	T/**S**
R01/99	G	A/**K**	G/**A**	C	A/**T**	C	G/**R**	T/**S**
H02/99	A	A/**K**	G/**A**	C	G/**A**	T	G/**R**	T/**S**
H20/03	A	G/**E**	G/**A**	T	A/**T**	C	G/**R**	T/**S**
R31/04	A	G/**E**	G/**A**	C	A/**T**	C	G/**R**	C/**P**
N32/04	A	G/**E**	G/**A**	C	A/**T**	T	G/**R**	T/**S**

positions) and the isolate SAVR01/99 residing in the KATRS group. Members of the EATRS group share an identical amino acid sequence in the investigated genome region despite a considerable geographical dispersal. Isolates belonging to this group were collected in the years 2002 to 2004 from a geographically

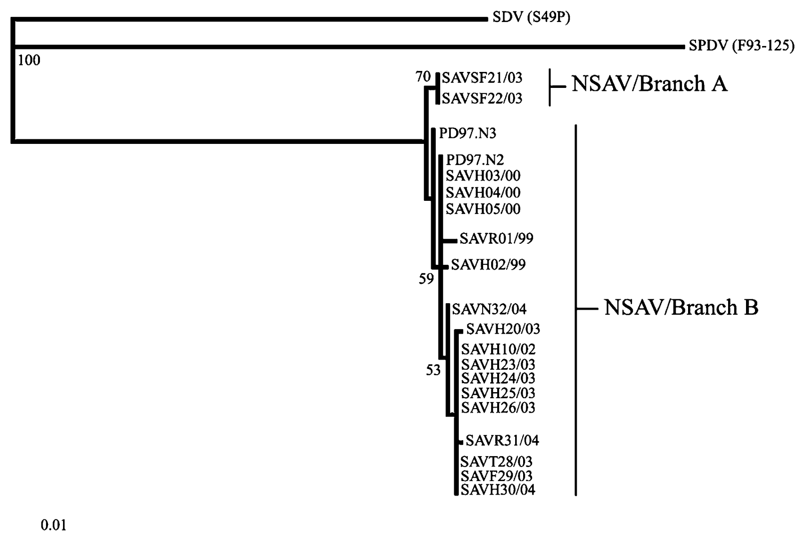

Fig. 2. Phylogenetic relationships among SAV isolates. SDV (S49P) and SPDV (F93-125) reference isolates are used as outgroups. The tree was bootstrapped using 1000 replicates, and bootstrap values <50 are not shown

Table 4. Substitution rates with 0.95 confidence limits for surface protein genes of selected alphaviruses and fish viruses generated through maximum likelihood and the single rate dated tips (SRDT) model. Validation of a clock-like evolution is indicated

Virus	Gene	Nt subst/site/year	Mol. clock	Source
Togaviridae				
EEEV	26S	$2.0\ (1.6, 2.6) \times 10^{-4}$	N	[17]
Highlands J	E1	$1.4\ (0.86, 2.6) \times 10^{-4}$	Y	[17]
WEEV	E1	$0.55\ (0.15, 1.6) \times 10^{-4}$	N	[17]
SAV subtype NSAV	C-E3-E2	$1.7\ (0.67, 2.73) \times 10^{-4}$	N	Present study
Orthomyxoviridae				
ISAV EU-G2	HE	$1.44\ (1.09, 1.79) \times 10^{-4}$	N	[30]
ISAV EU-G3	HE	$2.15\ (1.47, 2.83) \times 10^{-4}$	N	[30]
*Rhabdoviridae**				
VHSV Ia	G	$1.74\ (1.42, 2.06) \times 10^{-3}$	Y	[10]
VHSV Ib-IV	G	$7.06\ (6.61, 7.57) \times 10^{-4}$	Y	[10]

*Substitution rates and validation of a molecular clock for VHSV are based on substitutions per codon and given as nt subst/codon/year [10]

widespread area including counties Finnmark, Troms, Nordland, Sogn og Fjordane, and Hordaland. The KATRS group contains identical sequences collected from counties Hordaland and Rogaland during four years (1997–2001).

Maximum likelihood analysis confirmed that the NSAV isolates are very closely related and separate distinctly from the SDV and SPDV subtypes (Fig. 2). Although low bootstrap values were obtained within the NSAV subtype, the two Sogn og Fjordane isolates seem to belong to a slightly different lineage than the rest of the isolates (referred to as branch A and B, respectively). This was the only branching supported by an acceptable bootstrap value. A substitution rate of 1.70 $(\pm 1.03) \times 10^{-4}$ nt subst/site/year with 0.95 confidence intervals (Table 4) was obtained through maximum likelihood and the single rate dated tips (SRDT) model [36]. The SRDT model produced a tree that was significantly worse than the DR model ($p<0.05$). A molecular clock was therefore rejected for this dataset. Excluding branch A from the calculations yielded a similar result (available upon request).

Cell culture studies

The isolate SAVH20/03 was cultured through 20 passages in CHSE-214 cells. No cytopathic effect (CPE) could be detected during the first 12 passages. However, five days into the 13th passage, a CPE was observed. The CPE could be seen as curled up and vacuolated cells with pseudopodia like extensions (Fig. 3) and began in foci and increased to a web of affected cells throughout the monolayer as the infection continued. An increased amount of dead cells was observed in the supernatant. The CPE was observed in all of the following passages and occurred as early as one to two days after infection in passage 20.

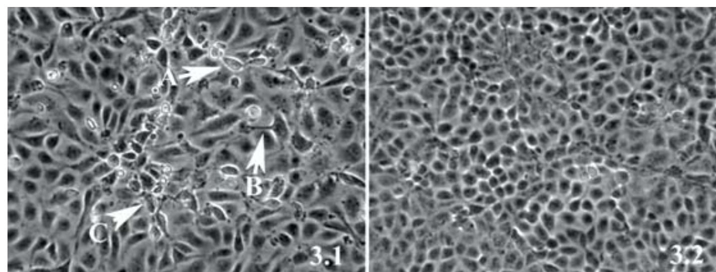

Fig. 3. CPE in CHSE-214 cells caused by NSAV infection. Figure 3.1 and 3.2 show cells after 13 passages of NSAV infection and uninfected control cells, respectively. NSAV CPE is recognized as curled up cells (**A**), pseudopodia-like extensions (**B**), and vacuolization in the cytoplasm (**C**)

Table 5. Substitutions that occurred during 20 passages of NSAV in CHSE-214 cells

Passage	Gene	Position within gene		Substitution	
		Nucleotide	Amino acid	Nucleotide	Amino acid
7	nsP2	1103	368	A to G	R to K
20	nsP3	1582	528	C to T	C to R
13	E2	616	206	T to C	S to P
19	E2	1124	375	C to T	I to T

Almost the entire genome (11530 nt) was sequenced after 20 passages and aligned with the passage three sequence (accession no. AY604235). A total of four nucleotide differences were observed (Table 5). Sequencing of selected regions of the genome revealed that one of these substitutions, the serine to proline substitution in E2 position 206, occurred in passage 13, at the same time as the appearance of CPE.

Discussion

Phylogenetic studies

The present study found only one subtype of SAV circulating in Norway. This subtype is clearly distinct from the SDV and SPDV subtypes (Fig. 2) that have been reported to cause disease in France, Ireland, and the UK [14, 42, 52]. The results are in agreement with those of Hodneland et al., who reported that SAV causing disease in Norway belongs to a genetically distinct subtype, NSAV [14]. The NSAV subtype was found to be genetically homogenous, although the isolates covered a large geographical area over a period of eight years. Despite the lack of diversity, it was possible to identify one slightly distinct branching with an acceptable bootstrap value (branch A) (Fig. 2). Branch A was found in southern Sogn og Fjordane, causing disease in two Atlantic salmon farms in 2003, and represents the northernmost part of what traditionally has been regarded as the enzootic area of NSAV.

Branch B, containing the rest of the NSAV isolates, seems to be widespread within the enzootic area in western Norway. It is surprisingly homogenous; less than 0.5% separates the isolates within the branch, and identical nucleotide sequences have been isolated from the years 1997 to 2001 and from the years 2002 to 2004. The great genetic homogeneity may justify the conclusion that sequences in branch B are temporally distinct samples of the same virus reservoir. The fact that the lower confidence limit of the calculated NSAV substitution rate was higher than zero (Table 4) should further defend such a conclusion, since regarding the isolates as temporally distinct sequences significantly increases the likelihood of the phylogenetic tree [17, 36]. It is intriguing that SAV-caused disease, until recently, has been a problem only in a very restricted area of Norway. As this study has shown, it is believed that these disease problems are caused by a very homogenous virus reservoir. The genetic homogeneity can only be explained by either extensive geneflow within the virus reservoir or a common source of virus. Similarly high degrees of genetic homogeneity within enzootic foci have previously been reported for terrestrial alphaviruses and are expected to reflect isolated host populations and viral geneflow within them [4, 20, 22, 24, 31, 38, 40, 44, 47, 50]. More information concerning the natural host spectrum of SAV is needed, however, in order to understand the apparent geographical restriction of the virus to western Norway. The isolates from the 2003 and 2004 outbreaks in northern Norway also belong to branch B and are very similar or identical to the Hordaland isolates (Table 3). NSAV has previously never been reported outside the enzootic focus of western Norway, and these areas are separated by at least 800 km. Thus, it is possible that human activity has transported the virus to northern Norway. The suspicion is strengthened by the fact that the three incidents in northern Norway involve smolts that have been transported from Hordaland county or fish that have been co-cultivated with Hordaland smolts that later developed PD (pers. obs.). It is not clear, however, whether the smolts were infected in the freshwater or the early saltwater phase.

NSAV and SPDV have traditionally been believed to utilize marine reservoirs [18, 26], but SAVR01/99 was collected from freshwater smolts [29]. SAV occurring in freshwater is not only restricted to the SAVR01/99 isolate, since there have been other findings of NSAV from Atlantic salmon fingerlings (pers. obs.), and since the SDV subtype affects reared rainbow trout in freshwater farms in France and the UK [11, 42]. In addition, the island-like distribution of the three SAV subtypes and the seemingly strict limitation of the enzootic NSAV area are likely to reflect isolated host populations. A working hypothesis is therefore suggested in which transmission of the virus occurs mainly in freshwater. In this phase of the Atlantic salmon lifecycle, genetically distinct populations are concentrated in rivers, in contrast to the marine phase, in which Atlantic salmon populations from the entire northern Europe intermingle in the north Atlantic [12, 16]. Little or nothing is known, however, concerning SAV in natural populations of salmonids, and the data presented thus far are therefore not extensive enough to draw any conclusions.

The substitution rate of NSAV was calculated through maximum likelihood and the SRDT model [36] to be 1.70 ($\pm$1.03) $\times 10^{-4}$ nt subst/site/year. The

molecular clock was rejected, and a reconstruction of evolutionary events based on the obtained rate would therefore be less informative. The NSAV substitution rate is low compared to those generally reported from RNA viruses, and this seems to be a common trait with most alphaviruses [5, 17, 38, 40, 44, 46, 47]. Jenkins et al. presented substitution rates derived from 50 RNA viruses through maximum likelihood and the SRDT model. The authors concluded that although not obvious, the rates of arthropod-borne (arbo) viruses were significantly lower than those of non-arboviruses [17]. The two-host lifecycle of alphaviruses has been postulated to provide a stabilizing selection, thereby constraining the evolutionary rates of these viruses [45]. The substitution rate of NSAV is approximately five to ten-fold lower than those of most non-arboviruses, which could be a reflection of stabilizing selection provided by an invertebrate vector host involved in transmission. However, low and varying substitution rates have also been reported for non-arbo fishviruses viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV) and infectious salmon anaemia virus (ISAV) [10, 21, 30], and a low evolutionary rate alone can therefore not be regarded as evidence for the existence of an invertebrate vector. *Lepeophtheirus salmonis* and *Caligus elongatus* are parasitic arthropods of salmonid fish that have been mentioned as hypothetical vectors of SPDV [51, 52]. NSAV has been detected by real-time PCR in *L. salmonis* collected from diseased fish (pers. obs.), but it is not clear whether the source of the virus was poorly digested blood or tissue from the salmon, or virus replicating in louse tissue.

The low substitution rate merely suggests that a mechanism exists to stabilize the molecular evolution of NSAV. The nature of this mechanism, however, remains unidentified.

Genetic stability in CHSE-214 cells

The NSAV consensus sequence is considered to be relatively stable in CHSE-214 cells. The sequence comparison of passage three and passage 20 revealed that only four nucleotide substitutions had occurred in the 11530 nucleotides that were sequenced. It is unusual and noteworthy however, that all these substitutions led to amino acid substitutions, which may have had an effect on the biological properties of the virus. CHSE-214 cells are derived from *O. tshawytscha* and do therefore represent a new host species for the SAVH20/03 isolate. This, together with the relatively low moi that was used during passaging of the virus, may have caused a genetic drift effect or selection for new phenotypes. Whether the four consensus substitutions merely represent a change in the quasispecies equilibrium or actual mutations introduced to the population during culturing can not, however, be answered at this point. The rate of substitution observed for NSAV in CHSE-214 cells resembles the observations of genetic stability in cell culture for the terrestrial alphaviruses Ross River virus (RRV) and Eastern equine encephalitis virus [38, 48]. In regard to CPE, it was absent through 12 passages, and appeared first in passage 13. It seemed to increase in severity and distinctness through passages 13 to 20, appearing as rapidly as two days p.i. in passage 20. The

late CPE, together with the four amino acid substitutions, suggests that the virus adapts to grow in cell culture and that some properties of the virus population may have changed during culturing.

Comprehensive studies of terrestrial alphaviruses have shown that the E2 protein is involved in cell culture adaptation. It has been proven that the affinity for heparan sulphate is changed in cell-culture-adapted strains of Sindbis virus, RRV and Venezuelan equine encephalitis virus (VEEV), and the change in affinity has been associated with single amino acid substitutions [1, 6, 8, 13, 19, 37]. Two of the substitutions that were found in the NSAV passage 20 sequence resided within the E2 gene. One of these substitutions, the position 206 serine to prolin substitution, is located in an area of E2 that has been proposed to be involved with heparan sulphate affinity and cell culture adaptation of VEEV (position 209) and RRV (position 218) [1, 13]. Although it could have been caused by pure coincidence, it is noticeable that the E2 position 206 serine-to-proline substitution occurred in passage 13, at the same time as the appearance of CPE. It is also interesting that this substitution is likely to have evolved at least twice among wild-type isolates of NSAV (it is found in isolates SAVSF21/03, SAVSF22/03, and SAVR31/04) (Table 3, Fig. 2). Experimental challenge of fish with the different passages of SAVH20/03 has not been conducted. Such studies could provide more information concerning the effect of these substitutions on the *in vivo* virulence of the virus. This is of special interest regarding the many experimental transmission studies that have been based on cell culture isolates [3, 23, 25, 43, 51].

Conclusions and future perspectives

It is concluded that a genetically homogeneous NSAV population causes disease on the west coast of Norway. The virus is not believed to be enzootic in northern parts of Norway despite the recent reports of disease in this part of the country. Transportation of fish from the west coast is a likely source of these outbreaks. A low substitution rate suggests that a stabilizing evolutionary mechanism exists. The mechanism of maintaining NSAV in the restricted enzootic area of western Norway has not been identified, and mechanisms of transmission are therefore an issue that would be crucial to investigate further in order to improve management of the disease. This includes the possibility of vertical transmission and identification of possible natural reservoirs in Norway.

It has been confirmed that NSAV changes in CHSE-214 cells. However, only 4 amino acid substitutions were identified when a 11530-nt-long segment was sequenced after 20 passages and compared with a passage three sequence, suggesting that the changes occur at a relatively low rate. Whether these changes have an *in vivo* effect should be further investigated.

Acknowledgement

The authors would like to thank Dr. Karen Elina Christie at Intervet Norbio AS, Bergen, Norway, for supplying virus for cell culture studies. This work was founded by Norwegian Research Council grant 143286/140.

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