Salmonid Alphavirus (SAV)

- Genetic characterisation of a new subtype, SAV3, and implementation of a novel diagnostic method

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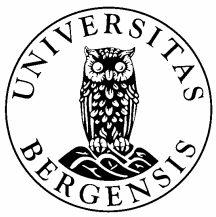

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CONTENTS

List of papers

This thesis is based on the following papers, hereafter referred to in the text by their Roman numerals:

Paper I

Hodneland, K., Bratland, A., Christie, K.E., Endresen, C. and Nylund, A., 2005. New subtype of salmonid alphavirus (SAV), Togaviridae, from Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* in Norway. Dis Aquat Organ 66, 113-120.

Paper II

Karlsen, M., Hodneland, K., Endresen, C. and Nylund, A., 2006. Genetic stability within the Norwegian subtype of salmonid alphavirus (family Togaviridae). Arch Virol 151, 861-874.

Paper III

Hodneland, K. and Endresen, C., 2006. Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan®). J Virol Methods 131, 184-192.

1 INTRODUCTION

Background

Since the onset of large-scale commercial salmon farming in Norway in the 1970-ies the industry has more or less continuously been hampered by "new" emerging diseases. As history has shown diseases originally with unknown aetiology, are in fact old pathogens that must have existed in nature long before salmonids were commercially domesticated. For instance ISAV, first reported in 1984 (Thorud, 1991), was initially called Bremnes syndrome and there were speculations on a bacterial aetiology (Hitra disease) or possible malnutrion. Years later, in 1993, final evidence for a viral aetiology was established (Watanabe et al., 1993). Also pancreas disease (PD), the pancreatic disorder first described from Scottish salmon (Munro et al., 1984), had an unknown aetiology for many years until the virus was isolated in by Nelson et al (1995). Although an infectious agent was suspected there was also some discussion on whether PD was a nutritional deficiency disease related to low Vitamin E and/or selenium (Bell et al., 1987; Ferguson et al., 1986b; Munro et al., 1984; Raynard et al., 1991; Rodger, 1991).

In the aquaculture industry at least two contributing factors are responsible for the enzootics observed for many of the diseases in fish; firstly, the naturally occurring pathogen have, through the high stocking densities of hosts occurring in intensive rearing, been given optimal conditions for replication and transmission and thereby have the potential to reach epizootic proportions. Secondly, any unintentional introduction of the pathogen(s) to naïve hosts or areas, by for example transport of infected hosts or otherwise infected material, can have detrimental effects on the newly exposed population of fish. Thus, a crucial measure in the prophylaxis of pathogens is to avoid introducing pathogens to farm sites via transport of new fish stocks that are put into production. One way of achieving this would be to test the fish-

stock for a particular pathogen before importing the fish into the facility. Other general preventive measures to reduce the importance of pathogens in a fish farm include vaccination whenever possible, regulations on transport and distribution of fish, slaughter and quarantine regulations, as well as sound farm management with good hygiene in order to reduce stress and/or physical damage to the fish resulting from unnecessary handling or transport. Today, efficacious vaccines are available for many of the bacterial pathogens in the salmon farming industry. The same success with viral fish vaccines has not been accomplished, and commercially available vaccines against infectious pancreas necrosis virus (IPNV), infectious salmon anaemia virus (ISAV), infectious haematopoietic necrosis virus (IHNV) and salmonid alphavirus (SAV) have considerable limitations in terms of protection and applicability (Sommerset et al., 2005). Especially IPNV and ISAV have been considered important viral pathogens in Norwegian salmon industry, but in recent years SAV has been recognized as a serious pathogen causing a dramatic increase in numbers of pancreas disease outbreaks. In the period from 1995 to 2004 a total of 137 farm sites were diagnosed with pancreas disease compared to 117 ISAV positive farms (E. Brun, National Veterinary Institute, Norway, pers. comm.). Despite that SAV has been known for more than ten years and has emerged as a serious threat to the salmon farming industry, our knowledge on the virus causing pancreas disease in Norway is very limited.

In the next sections some aspects regarding the general alphavirus biology are summarized following a review of the disease-causing alphavirus species in fish; Salmonid alphavirus (SAV), with emphasis on the Norwegian subtype of SAV.

The Alphavirus (*Togaviridae*)

The family *Togaviridae* consists of two genera; *Alphavirus* and *Rubivirus* (Schlesinger and Schlesinger, 2001). Their genomic organization is similar, but phylogenetic analyses have suggested that alphaviruses and rubiviruses are only distantly related (Koonin and Dolja, 1993). Rubella virus is primarily transmitted either through direct contact, inhalation of aerosol containing virus, or congenitally from mother to child. Alphaviruses on the other hand are typically transmitted by arthropod vectors, mainly by mosquitoes of Aedes and Culex families (Chamberlain, 1980), but also other haematophagous arthropods such as mites, bugs and ticks may function as vectors (Griffin, 2001). This two-host lifecycle gave rise to the historical classification of alphaviruses as arboviruses (arthropod-borne viruses). The alphaviruses use a wide variety of vertebrate hosts and are reported from all continents of the world except Antarctica. The genus Alphavirus contains at least 24 different species (Powers et al., 2001), some of which are responsible for important human diseases such as encephalitis ((Eastern (EEE), Venezuelan (VEE) and Western (WEE) equine encephalitis viruses)) or fever, rash and polyarthritis ((Chikungunya, O'Nyong- Nyong (ONN), Ross River and Sindbis (SIN viruses)) (Strauss and Strauss, 1994). Recently, a new species in the Alphavirus genus has been described from salmonid fish, for which the name Salmonid Alphavirus is proposed (Weston et al., 2002).

General Alphavirus structure

Members of the Alphaviruses are small (45 to 75 nm in diameter), enveloped viruses, and have an icosahedral nucleocapsid core surrounded by a membrane bilayer. The nucleocapsid consists of one copy the positive (+) single-stranded RNA genome complexed with 240 copies of the capsid protein. Individual capsid proteins are arranged as pentamers and

hexamers to form a T=4 icosahedral symmetry (Cheng et al., 1995; Paredes et al., 1993). This symmetry is also maintained for the viral glycoproteins embedded in the lipid bilayer surrounding the nucleocapsid. The lipid bilayer of the virion has a phospholipid composition that resembles that of the host plasma membrane, and anchored in this virion envelope are 80 copies of viral glycoprotein spikes (Figure 1). Each spike on the virus surface is composed of a trimer of two or three subunits; the glycoproteins E1 and E2 $(E1/E2)_3$, and in some alphavirus species an additional peripheral protein E3 $(E1/E2/E3)_3$. The latter subunit is normally extremely efficiently cleaved and released from the E2 precursor protein (PE2),

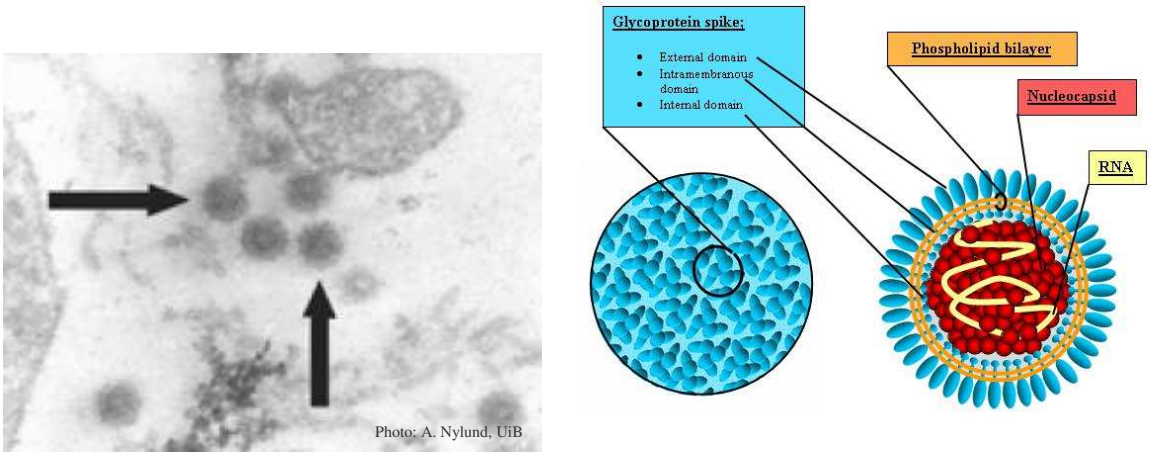

Figure 1. Left: Electron micrograph image of *Salmonid alphavirus* particles (arrows). Middle: Schematic reconstruction of an Sindbis virus indicating the arrangements of the glycoprotein spikes. Right: Cross-section representation of Sindbis virus with the glycoproteins (E1 and E2), the phospholipid bilayer, nucleocapsid, and RNA.

thus rendering the mature virus particle free of E3. E1 and E2 form a stable heterodimer, and three copies of these E1-E2 heterodimers are intertwined to form one spike. The virus contains 240 heterodimers, and these are assembled into 80 spikes organised into the T=4 icosahedral surface lattice (Cheng et al., 1995; Fuller, 1987; Fuller et al., 1995; Vogel et al., 1986).

The carboxy-termini (-COOH) of the E1 and E2 membrane spanning anchors interact with the capsid, while the amino termini of both E1 and E2 face outward from the lipid membrane. In addition, a small hydrophobic viral protein called the 6K is associated with the membrane. Although 6K is expressed from the same open reading frame (ORF) at equal rates as the capsid, E3, E2 and E1, it is associated with the virus in low quantities from 7 to 30 molecules per virus particle (Gaedigk-Nitschko and Schlesinger, 1990; Lusa et al., 1991). The exact role of 6K is not fully understood, but it is believed to be a virally encoded ion channel protein (viroporin) (Melton et al., 2002) that has been shown to affect glycoprotein processing, transport of proteins through the ER, and virus budding (Loewy et al., 1995; Sanz and Carrasco, 2001; Sanz et al., 2003; Yao et al., 1996).

Replication cycle of alphaviruses

Alphaviruses enter the cell by receptor-mediated endocytosis (RME), and are delivered intact into endosomes (Helenius et al., 1980; Kielian et al., 1986) (Figure 2). Since the alphaviruses have a wide host range and are capable of replicating in many different cell types, the interaction with a receptor on the surface of the target cell must involve either many types of protein receptors, and/ or one ubiquitous molecule on the surface of host cells. The highly conserved laminin-receptor found in mammals, birds and mosquitos has been recognized as a high-affinity receptor used by alphaviruses. Other known cell-receptors for alphavirus attachment include two surface-proteins (74-kd and a 110-kd) found on neuroblastoma cells of mouse, and the heparan-sulphate proteoglycan receptor found on most cell types. It appears that the E2 glycoprotein of alphaviruses is responsible for the receptor binding to cells, and that E1 only plays a limited role (Cheng et al., 1995). Studies from Sindbis virus have shown that important neutralizing epitopes reside in a domain between aminoacid residues 170 to 220, and that this domain interacts directly with cellular receptors (Strauss and Strauss, 1994).

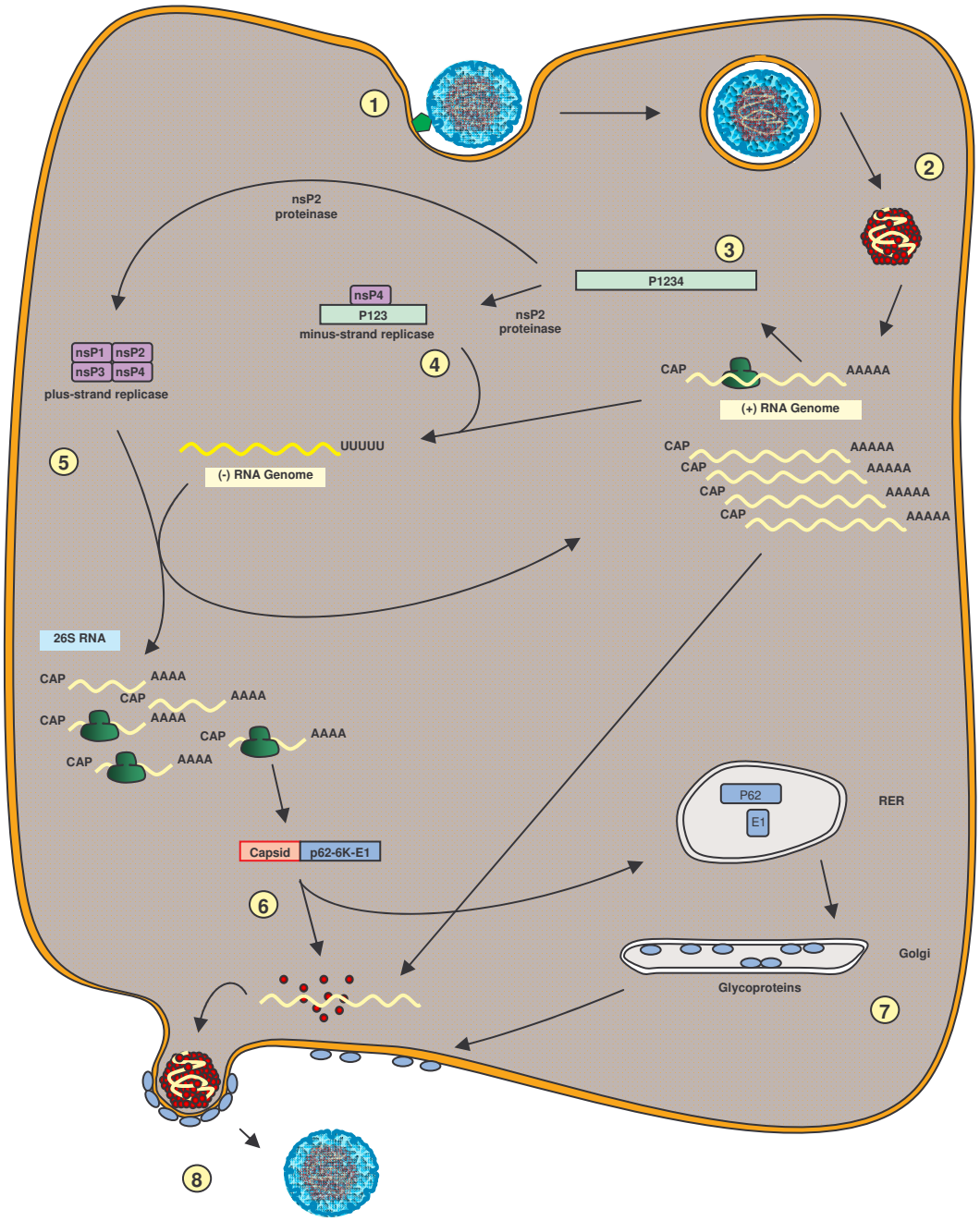

Figure 2. Replication cycle of Alphavirus (see main text for details); 1, The virus particles enter the cell via receptor-mediated endocytosis mediated by E2 and become internalized in endosomes. 2, The lowering of the pH in the endosomes triggers the membrane fusion activity of E1, allowing the release of the nucleocapsid into the cytoplasm. 3, The 49S (+) RNA genome binds to ribosomes, resulting in the synthesis of the nonstructural polyprotein (P1234). 4, Autoproteolytic cleavage of P1234 produces the replicase complex P123-nsP4 which transcribes the genome into full-length 42S minus-strand RNA-templates. 5, Only 3-4 hours after infection the cleavage of P123 is accelerated as a result of the accumulation of P123-nsP4 in infected cell, producing four mature proteins nsP1- 4. Then the minus strand production ceases and the newly formed replicase complex nsP1-4 produces only plus-strand RNAs (49S and 26S). 6, The subgenomic 26S RNA is translated into the structural proteins as a polyprotein consisting of capsid-P62-6K-E1. The capsid is autoproteolytically cleaved off in the cytosol, and the remaining polyprotein is translocated to the lumen of the ER. 7, After binding to carbohydrate chains the polyprotein is cleaved by signalases into p62, 6K, and E1. The p62 and E1 proteins associate into heterodimers which are transported to the Golgi complex and transferred to the plasma membrane. 8, After assembly of the capsid and viral genomic RNA the nucleocapsid bind to the glycoproteins at the plasma membrane, initiating the budding process.

Once the virus is bound to its cell surface receptor, it accumulates in coated pits which become endocytosed and internalized in an endosome (cf. Strauss and Strauss, 1994). The viral envelope then fuses with the endosome membrane, and the nucleocapsid (NC) is released into the cytoplasm. This fusion process is hypothesised to be pH-dependent, and to require the presence of cholesterol on the target membrane. The lumen of early endosomes become mildly acidic, and it has been shown that this low pH triggers conformational changes in the viral spike proteins. More specifically the E2/E1 heterodimer dissociates when the pH is lowered (Wahlberg and Garoff, 1992) and E2 moves away. As a result, the position of the E1 is altered somewhat so that it facilitates the interaction with cell surface components via its fusion domain. The putative fusion domain in E1 is believed to reside in a highly conserved, hydrophobic region between residues 78 and 98 (cf Strauss and Strauss, 1994). Following the dissociation of the E2/E1 heterodimer the E1 becomes trimerized, and it is postulated that groups of five copies of the homotrimerized E1 will force the two opposed membranes (virus envelope and endosome membrane) together (Gibbons et al., 2003; Gibbons et al., 2004).

After fusion of the two membranes the nucleocapsid enters the cells cytoplasm and dissociation of the nucleocapsid starts almost immediately. It is proposed that the trimerization process of the E1 subunits leads to pore formation in the membrane of the mildly acidic endosomes, and that the influx of protons through the pores forces the capsid protein to undergo a structural change. The conformational change primes the nucleocapsid for final disassembly by interactions with the capsid ribosome-binding site and the ribosomes (Lanzrein et al., 1994; Mrkic et al., 1997).

Once released into the cytoplasm the alphavirus genome binds to ribosomes and serves directly as the messenger RNA for protein synthesis, and as a template for the synthesis of the complementary 42S minus strand (Figure 3).

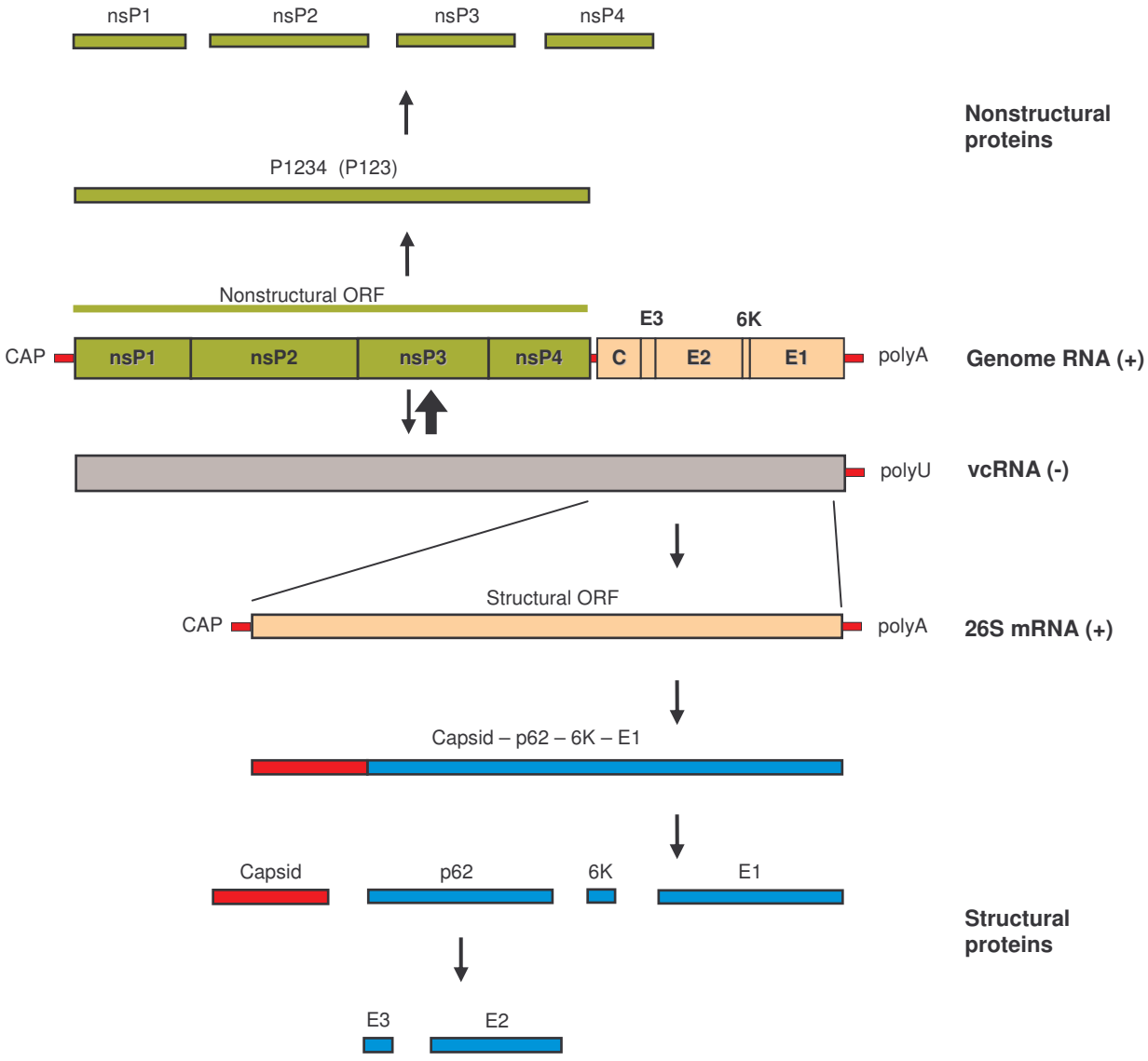

Figure 3. A schematic alphavirus genome organization. (See text for details). The 5' two thirds of the genome codes for the nonstructural proteins nsP1-4, which are directly translated and processed from the plus-strand genome. The complementary minus-strand of the viral genomic RNA (vcRNA) is synthesized by a P123-nsP4 replicase complex, and serves as a template for the transcription into a 26S subgenomic mRNA. vcRNA is also a template for the generation of new plus-strand genomic RNA by the action of a nsP1-4 replicase complex. Translation of the 26S mRNA results in a polypeptide consisting of capsid-p62-6K-E1. Enzymatic processing of the polypeptide produces the structural proteins capsid, E3, E2, 6K and E1.

The read-through of the 5' two thirds of the 42S alphavirus genome is translated into a single polyprotein P1234 which is autoproteolytically cleaved, by function of nsP2, into a replicase

complex consisting of P123 and nsP4. These proteins form an RNA-dependent RNA polymerase complex that transcribes the genome into full-length 42S minus-strand RNA-templates. Three to four hours after infection, the build-up of proteinases in the cell renders this replicase complex unstable, and the P123 is further cleaved into nsP1, nsP2 and nsP3. The resulting nsP1-4 now constitutes a highly efficient replicase complex that only produces (+) strand RNAs (cf Strauss and Strauss, 1994).

A full-length 42S minus strand serves as template for the synthesis of the subgenomic 26S mRNA, which corresponds to the last one third of the genome. The 26S RNA encodes the viral structural proteins; capsid, E1 through E3 and 6K. This structural domain is transcribed as a polyprotein consisting of capsid-P62-6K-E1. The capsid protein is autoprotelytically cleaved from the polyprotein, and rapidly associates with genomic 42S RNA in the cytoplasma to form icosahedral nucleocapsid structures (cf Garoff et al., 2004; Strauss and Strauss, 1994). A signal sequence on the remaining p62-6K-E1 results in the translocation of the polypeptide to the lumen of the rough endoplasmic reticulum (Garoff et al., 1990; Garoff et al., 1978). Here, the polypeptide is modified by covalent attachment of oligosaccharides, and later proteolytically cleaved into p62, 6K, and E1 (Liljestrom and Garoff, 1991). The p62 and the E1 proteins interact to form heterodimeric complexes in the ER, and are then transported to the Golgi complex. After transport through the Golgi complex the glycoproteins are delivered via the secretory pathway and accumulate in the plasma membrane of the host cell. During the transport via the Golgi network, but before the appearance at the plasma membrane, p62 is already oligomerized into E2 and E3 (de Curtis and Simons, 1988). The cytoplasmic nucleocapsid are thought to diffuse freely to the sites of the plasma membrane where the viral glycoproteins are embedded. There the cytoplasmic C-terminus of the E2 in the glycoprotein spike bind in a 1:1 molar ratio to the newly arrived nucleocapsids, and initiates the final assembly and budding of new viruses will occur. Also,

lateral interactions between glycoproteins are essential for an effective budding of virus. It has been proposed that the nucleocapsid-E2 binding triggers the spikes to interact laterally with each other, and that these spike-spike interactions are responsible for the viral envelope formation (Garoff and Cheng, 2001). As the number of bindings between nucleocapsids and glycoproteins increase, the glycoprotein-containing membrane become tightly pulled around the nucleocapsid until the whole particle is surrounded with the membrane and finally buds off (Garoff et al., 1998).

Evolution of RNA viruses

The success of RNA viruses as intracellular parasites is largely due to their simplicity and small size, but most important is their ability to quickly respond and adapt to changing environments. The reason for their adaptive strength is coupled with the high substitution rates, short replication times, and large population size potential. RNA viruses have the highest substitution rates found in nature ranging from 10^{-3} to 10^{-5} misincorporations per nucleotide copied (Drake and Holland, 1999). The high rate of spontaneous substitution is thought to be a result of absence of proofreading activities of RNA replicases and retrotranscriptases (Steinhauer et al., 1992). Together with the short replication times and usually large population sizes, the RNA virus population will consequently consist of a complex collection of genomes with different substitutions rather than as copies of one or a few dominant sequences. The sequence diversity will then consist of the single master RNA genome sequence, plus all the different mutants in the population. This complex dynamic entity is often referred to as a ‘‘quasispecies’’ (Domingo et al., 2001) (Figure 4).

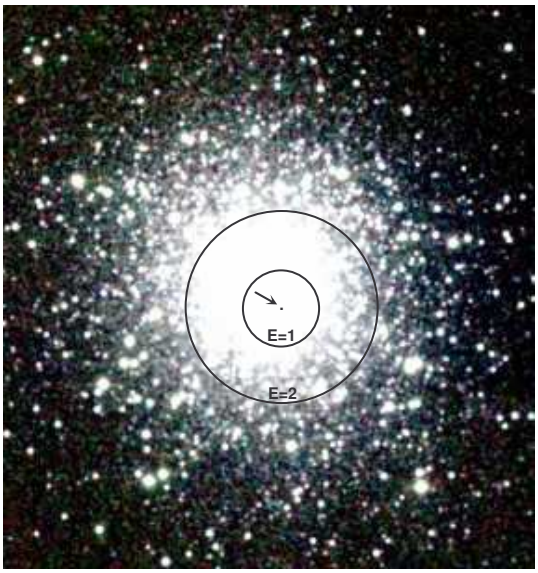

Figure 4. This picture of a globular star cluster can be used as an analogy to exemplify the concept of the quasispecies. If each point in regular 3-dimensional space corresponds to a genome sequence, then the sum of all stars represent the collection of genomes that form a complex RNA population. At the centre of the cluster is the master sequence (arrow). Immediately surrounding it are sequences with 1 error. Sequences with 2, 3, and more errors are progressively farther out. (Modified from: *http://www.microbiology.wustl.edu/dept/fac/huang/ccas/mut/mut.html#m13*)

Despite the fact that RNA viruses may have a quasispecies distribution which constantly generates new mutants, the master genome is maintained at a stable frequency in the population during passaging in in-vitro systems (such as cell-culture). This is because advantageous mutants will continue to replicate faster than deleterious ones as long as the environmental conditions (cell-culture) remain stable (Steinhauer and Holland, 1987). This explanation for the maintenance of the master sequence in culture may also apply to evolution in nature. Only those features that are the most strongly selected for under a variety of environmental conditions will remain conserved. The frequency of any mutant in the quasispecies is determined by its own replication success, as well as the probability that it will arise by the erroneous replication of other mutants in the population. The replication success in turn is governed by selective forces during changing environmental conditions, and the quasispecies is thought to evolve towards an equilibrium of mutation-selection processes which maximize the average rate of replication of the mutant spectra as a whole. As a consequence of this huge collection of genome variants, a mutant of initial lower fitness may

possess a selective advantage over the master sequence when the environmental conditions change, and will thus become the dominant species. Changing environmental conditions may be exposures to different host species or cell types, and various immune responses (inflammatory action, interferons). Although much cited, there are contradicting views on whether the quasispecies concept is a meaningful theory of RNA virus evolution compared to conventional population genetics. However, according to Wilke (2005) there are no real contradictions between the two, and he concludes that the quasispecies theory is perfectly equivalent to the concept of mutation-selection balance developed in population genetics. A mutation- selection balance states that the deleterious genetic variant in an infinite population will reach an equilibrium between the rate at which the mutant gene arises by recurrent mutation, and its elimination by natural selection.

Despite the high substitution rates in RNA viruses the evolutionary rates may vary considerable, ranging from 10^{-2} to $<10^{-6}$ nt substitutions per site per year. Slow rates of evolution seem to be a general feature among arthropod-borne viruses, which has been attributed to stabilizing selection for successful replication in both the vertebrate host and the invertebrate vector (Weaver et al., 1992). There are however arboviruses such as the North and South American EEEV which have a non-uniform evolutionary rate. Possible explanation for the increased rate in some EEEV lineages involve changes in virus dispersal and population sizes due to fluctuations in the vertebrate host and/or invertebrate vector, or other rapid evolutionary changes such as genetic bottlenecks or founder effects. (Weaver, 1995).

Alphaviruses in fish

Today, the only alphavirus species known from fish is the Salmonid alphavirus (SAV), which can be divided into three subtypes; SPDV/SAV1 (Weston et al., 1999), SDV/SAV2 (Villoing et al., 2000a) and NSAV/SAV3 (**Paper I**).

The first concrete evidence for an Alphavirus in fish was presented from Ireland by Weston et al., 1999). Cloning and sequencing of a 5.2 kb fragment of a virus isolate from salmon suffering from PD, demonstrated a gene organization and sequence similarity which agreed with an alphavirus aetiology. This milestone in the SAV research was published 23 years after PD was first recognized in Scotland in 1976 (Munro et al., 1984). Many new records and descriptions of PD were published in the following 10 years from Scotland (Ferguson et al., 1986a; Ferguson et al., 1986b; McVicar, 1987; McVicar, 1990), Ireland (Murphy et al., 1992; Rodger, 1991), North America (Kent and Elston, 1987), and Norway (Poppe et al., 1989). Depending on which clinical signs and histopathological lesions that were most prominent in the examined tissues in these studies, the disease has been given different names such as exocrine pancreas disease (Munro et al., 1984), polymyopathy syndrome (PMS) (Roberts, 1989) or sudden death syndrome (SDS) (Rodger, 1991). These are all thought to describe the disease now commonly referred to as pancreas disease or PD, although the pancreas lesions itself are not always the most significant histopathological finding.

Parallel to this, a disease with similar histopathology was described from freshwater reared rainbow trout in France. The name sleeping disease was given due to the striking behaviour where diseased fish rest on their side on the bottom of the tanks, but when handled start swimming for some time before returning to "sleep" (Boucher and Baudin Laurencin, 1994). The virus responsible for SD was isolated by Castric et al (1997), and the first nucleotide sequence (Villoing et al., 2000a) showed that the SD and SPDV virus were closely related. Historically, PD in Norway was believed to be caused by the same virus as in the British Isles (SAV1), but it is now accepted that the only SAV present in Norway is the newly characterized NSAV/SAV3 (**Paper I**; **Paper II**). Infections with SAV seem to be restricted to the two genera *Oncorhynchus* and *Salmo* (Table 1).

Table 1. Records of naturally occurring- or experimental infections with SAV from different fish hosts in either fresh- or seawater conditions.

	SAV1		SAV2		SAV3	
Host	Natural	Experimental	Natural	Experimental	Natural	Experimental
Salmo salar	Yes [b]	Yes [c]	Yes [a]	Yes [a]	Yes [c]	Yes [c]
Oncorhynchus mykiss	No	Yes [a]	Yes [c*]	Yes [a]	Yes [b]	No
Salmo trutta	Yes [b]	Yes [a]	Yes [a]	Yes [a]	No	No
Other	No	No	Yes [†]	No	No	No

[a] from freshwater
[b] from seawater
[c] from freshwater and seawater (* as "Summer lesion" in seawater reared rainbow trout (Baudin Laurencin et al., 1985))
[†] Coho salmon (Boucher, P. and Baudin Laurencin, F., 1994)

Molecular characteristics of SAV

The first molecular evidence of SAV came with Weston et al's (1999) cloning and sequencing of a 5.2 kb fragment of the virus (SAV1) previously isolated by Nelson et al (1995) in Ireland. The translated nucleotide sequence showed considerable organizational and sequence identity to the structural proteins from other alphaviruses. Later sequencing studies of SAV1, SAV2 and SAV3 confirmed the phylogenetic position of SAV as an alphavirus species (**Paper I**; Villoing et al., 2000a; Weston et al., 2002).

The nucleotide sequence identity of the three SAVs is above 90 % over the complete genome, while the similarity to the mammalian Alphaviruses is much lower (**Paper I**). As for all Alphavirus two open reading frames (ORF's) are also present in the SAV genome; first a continuous ORF encoding the four nonstructural proteins (nsP1-4) and a second ORF encoding the structural proteins (Capsid, E1-3 and 6K) (Table 2).

Table 2. Comparison of protein sizes (aa) for nonstructural and structural proteins in SAV.

Virus protein	SAV1	SAV2	SAV3
nsP1	562	561	561
nsP2	859	859	859
nsP3	571	564	558
nsP4	609	609	609
C	282	283	281
E3	71	71	71
E2	438	438	438
6K	68	68	68
E1	461	462	461

The first ORF is flanked at its 5' end by a 27 nt long nontranslated region (NTR) and a 35 nt long NTR at the 3' end, which immediately precedes the second ORF (Weston et al., 2002). The second ORF contains approximately 90 nt at its 3' end followed by a poly(A) tract. Full length sequences, excluding the poly(A) tracts at the 3' termini, consist of 11,919 and 11,900 nt for SAV1 and SAV2. The SAV3 sequence lacks approx. 8-53 nucleotides at the 5'end of nsP1 but is otherwise complete at 11,831 nucleotides.

A phylogenetic analysis of 11,700 nt from six different isolates of SAV clearly indicates that the salmonid alphavirus species constitute 3 different subtypes (**Paper I**) (Figure 5). This conclusion was later supported in a phylogenetic study by Weston et al (2005) on nt sequences from E1 and nsP4 gene fragments from SAV isolates originating from British Isles, France and Norway. A comparison of the nucleotide and amino acid sequence identities of the individual nonstructural and structural proteins for all SAVs are summarized in Table 3 and 4. The aa sequence differences between the three subtypes range from 97-98% for the nonstructural proteins, and 94.4-95% for the structural proteins. A pairwise comparison of SAV and selected members of the alphaviruses show that SAV is distantly related to all the established members of the genus *Alphavirus*; the average percentage amino acid identity of

SAV and other alphaviruses is 42.5% for the nonstructural and 32.5% structural proteins (Weston et al., 2002, present study). In general, the SAVs contain larger individual

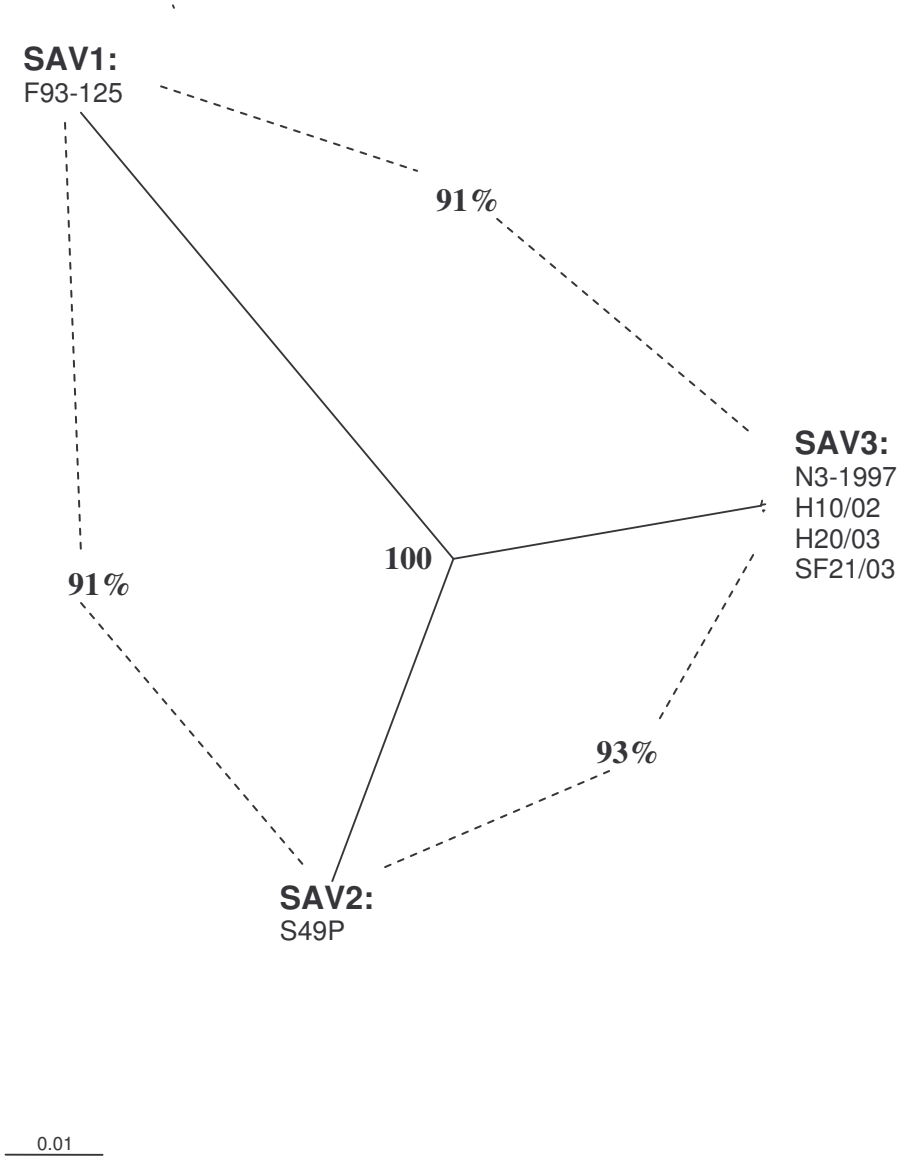

Figure 5. Salmonid alphaviruses (SAV). Genetic distance of the SAV subtypes in relation to each other. Evolutionary relationship based on alignment of complete genome (11720 nucleotides) of 6 SAV isolates including all 3 subtypes (SAV1, SAV2 and SAV3). Scale bar: number of nucleotide substitutions as a proportion of branch length. Percent nucleotide similarity between the subtypes is shown.

nonstructural and structural proteins compared to other alphaviruses, whereas within SAV there is very little variation. The only exception in this respect is nsP3 which is the most divergent gene with a number of nt substitutions. The mean aa identities are 95-96% for the nonstructural and structural proteins as a whole, but for nsP3 alone the aa identities is 91-93%

for the SAV subtypes (insertions/deletions excluded). In addition, the nucleotide lengths of nsP3 range from 1713, 1692 and 1674 nt in SAV1, SAV2 and SAV3, respectively.

Table 3. Salmonid alphavirus (SAV). Percent nucleotide (nt) sequence similarities between the 3 subtypes in Europe, comparing the different ORFs (open reading frame of SavH10/02 isolate) on the genomic strand.

		SAV3 (SavH10/02)								
Subtype	Isolate	nsP1*	nsP2	nsP3	nsP4	C	E3	E2	6K	E1
SAV3	N3-1997	99	99	99	100	99	99	99	99	100
SAV3	SavH20/03	100	99	99	99	100	100	99	100	100
SAV3	SavSF21/03	99	100	99	100	99	99	99	100	100
SAV1	F93-125	94	91	85	92	91	89	89	94	93
SAV2	S49P	95	93	88	94	88	92	92	94	93
	ORF/nt	1631*	2577	1674	1829	845	210	1316	206	1385

* A few nucleotides are missing at the beginning of the ORF

Table 4. Salmonid alphavirus (SAV). Percent amino acid (aa) sequence similarities between the 3 subtypes in Europe, comparing the different proteins.

		SAV3 (SavH10/02)								
Subtype	Isolate	nsP1*	nsP2	nsP3	nsP4	C	E3	E2	6K	E1
SAV3	N3-1997	100	99	99	100	100	98	99	98	100
SAV3	SavH20/03	100	100	100	100	100	100	100	100	100
SAV3	SavSF21/03	100	100	99	100	100	98	99	100	100
SAV1	F93-125	95	96	88	97	95	94	95	97	98
SAV2	S49P	97	97	90	98	88	95	94	95	96
	No. of aa	543	859	558	609	281	71	438	68	461

* A few aa are missing at the beginning of the protein

The alphavirus genome contains sequence elements and secondary structures that are important for replication of the genomic RNA and its encapsidation, as well as transcription of the subgenomic 26S RNA. The four conserved nucleotide sequence elements, CS 1-4, are believed to be crucial for the replication of alphaviruses, possibly as promoters in the

replication of viral RNA. The putative CS1 is found in the 5' NTR of SAV1 and SAV2, although the sequence similarity with other alphaviruses is low. CS2 is located within the nsP1 and consists of a 52 nucleotide sequence capable of forming two stem-loop RNA structures in all SAVs. It is proposed that the CS2 have an important role in the minus-strand synthesis of alphaviruses. CS3 is part of the junction region between the nonstructural and structural proteins, and act as a transcriptional promotor for the subgenomic mRNA. This 24 nt sequence is identical for SAV2 and SAV3, with SAV1 differing at only 1 nt. A conserved 19 nt region in the 3' nontranslated region has been identified in all SAVs, and is thought to represent the CS4 which serves as a promotor for the initiation of minus-strand RNA synthesis (Villoing et al., 2000a, present study).

For many alphaviruses the translation of the first open reading frame (ORF) stops at an opal termination codon (UGA) between nsP3 and nsP4, thus producing the translation product P123. However, read-through of this stop codon occurs during ~10-20% of the translation events, and will instead result in the incorporation of an additional aa-residue in the new translation product P1234 (Strauss and Strauss, 1994). None of the SAV subtypes have a stop codon in this position, and alignments of the nsP3 and nsP4 region from SAV and other alphaviruses show that this termination codon in SAV is replaced by a glutamine (**Paper I**; Weston et al., 2002). The lack of an opal stop codon is also described in other alphaviruses (SFV and ONNV), but here UGA is replaced by an arginine residue in the polypeptide P1234 (Levinson et al., 1990; Takkinen, 1986).

Several of the conserved aa-motifs in the structural and non-structural alphavirus proteins can also be identified in SAV. For the non-structural proteins these include motif I , II and IV in the nsP1, the -G-X-X-G-X-G-K-T- motif in the nsP2, and the conserved residues Cys482 and His552 within the cysteine protease domain in nsP2 (**Paper I**; Weston et al., 2002). For Sindbis virus, the characteristic catalytic triad amino acid residues H^{142}, D^{163} and S^{215}

constitute the serine protease active site in the nucleocapsid. A corresponding serine protease site is also present in the capsid of SAV, although the position of the H-D-S triad is slightly different (Villoing et al., 2000a). The consensus sequence of the putative autocleavage site in the capsid is also present, and is identical for all SAVs (-P-W$^{\downarrow}$T-). Host mediated cleavage of the p62 into E2 and E3 is proposed to be located within the consensus furin site -R-X-$^{R}/_{K}$-R$^{\downarrow}$X. The expected size of E2 is observed (approx. 50kDa) in both SAV1 (Welsh et al., 2000) and SAV2 (Villoing et al., 2000a), indicating efficient cleavage of the p62. In SAV2/SAV3 the p62 furin cleavage site is identified as -R-K-K-R$^{\downarrow}$X-, but is slightly different in SAV1 (-R-R-K-R$^{\downarrow}$X-). There are no N-linked glycosylation sites present in the E3 protein in SAV, one site in E2 at N_{319}, and one site at position N_{35} in E1. The SAV E2 protein also contains a putative transmembrane and cytoplasmic tail domain located near the carboxy end. The cytoplasmic tail domain contains two highly conserved cysteine (C_{431} and C_{432}) residues. A multiple sequence alignment of E1 identified the putative fusion domain in SAV, and showed high sequence similarities with other alphaviruses (Villoing et al., 2000a). Of particular interest is the replacement of two glycine residues in SAV ($G \rightarrow N_{94}$ and $G \rightarrow A_{102}$), which theoretically would shift the pH threshold for fusion to a more acidic range.

SAV pathology and diagnostics

The disease caused by infections with SAV; pancreas disease (PD), was originally described solely on the basis of exocrine pancreas pathology (Munro et al., 1984), which included vacuolisations and complete necrosis of acinar pancreatic cells with subsequent replacement by fibrotic tissue. The pathogenesis was divided into three phases (preacute, acute and postacute) based on the severity of the degenerative changes. Although the attempts to experimentally infect salmon and rainbow trout failed, they suspected a viral aetiology. It was also speculated that the observed pancreas pathology was a possibly result of selenium

deficiency. It soon became evident that pathologies associated with SAV infections were more extensive and complex than only the exocrine pancreas lesion reported by Munro et al (1984). Ferguson et al (1986) described severe degenerative myopathy in both heart and red skeletal muscle, and concluded that the extensive myocardial lesions were the most significant change associated with SAV diseased fish. Similar degenerative lesions were also observed in the oesophageal muscle and muscle fibres elsewhere in affected fish. However, the only consistent tissue lesion in SAV affected fish was considered by McVicar (1986, 1987) to be necrosis of the exocrine pancreas, and the significant myopathies reported by Ferguson et al (1986) was not always evident in his material. He thus concluded that "*total loss of the exocrine pancreas was the only tissue lesion always found in early stages of the disease and this remains the only reliable pathological index of PD*". This rigid diagnostic criteria by McVicar (1986; 1987) and/or Munro et al's (1984) use of exocrine pancreas necrosis as a sole diagnostic criteria for SAV disease was adopted by several authors in the following years (Boucher et al., 1995; Houghton, 1994; Houghton, 1995; Lopez-Doriga et al., 2001; Murphy et al., 1992; Pringle et al., 1992; Raynard and Houghton, 1993; Rodger et al., 1994). However, growing evidence from sequential studies on the histopathology of SAV disease from field samples and experimentally infected fish clearly demonstrated that the cardiac and skeletal muscle lesions are indeed significant findings in affected fish (Boscher et al., 2006; Boucher and Baudin Laurencin, 1996b; Castric et al., 1997; Christie et al., 1998; Desvignes et al., 2002; Ferguson et al., 1986a; Ferguson et al., 1986b; Graham et al., 2003b; Mccoy et al., 1994; McLoughlin, 1997; McLoughlin et al., 2002; McLoughlin et al., 1995; McLoughlin et al., 1996; Nelson et al., 1995; Poppe et al., 1989; Rodger et al., 1995). By excluding these important diagnostic criteria there is a significant chance of missing those fish still having various amounts of normal pancreatic acinar cells but nevertheless affected by the disease. Thus, fish in the acute phase with focal or diffuse pancreatic acinar cell necrosis, and fish in

the recovery phase with surviving or regenerated pancreatic acinar cells would be diagnosed as SAV-free. Clearly, this could have serious implication for the interpretation of the data on prevalence and severity of SAV in any study. This problem was addressed in a study from Boucher et al. (1995) who compared the susceptibility of rainbow trout, brown trout and Atlantic salmon to SAV1. From their infection trials only salmon could be diagnosed with SAV disease using the above criteria. However, both the rainbow trout and the brown trout evidently also became infected, and were significantly affected by the infection with SAV1, but since they had substantial amounts of intact pancreatic acinar tissue left SAV disease could per definition not be diagnosed.

In an attempt to standardize the diagnostic criteria for SAV1 disease, a summary of clinical signs, gross pathology and the range of histopathological features of infections with SAV1 in salmon in the British Isles was published by McLoughlin et al (2002). Here, it is acknowledged that it is a complex disease syndrome with varying degrees of pathology especially in the key organs exocrine pancreas, heart and skeletal muscle. The severity and distribution of lesions may vary but appear in a definite and consistent manner during the time course of an outbreak (acute, sub-acute, chronic and recovery). Clinical signs of SAV1 disease typically include lethargic fish staying close to the water surface near cage walls, with some fish resting or hanging on the side of the net-pens. Histopathological findings essentially involve different combinations of lesions in exocrine pancreas, heart and skeletal muscle. These histopathological lesions also applies to fish suffering from infections with SAV2 (sleeping disease, SD). The first publication on SAV2 briefly describes characteristic necrosis of the skeletal red muscle and inflammatory lesions in exocrine pancreas and heart of rainbow trout (Boucher and Baudin Laurencin, 1994). A more comprehensive study, where experimental crossinfections with SAV2 and SAV1 infected material in rainbow trout, demonstrated that the difference between SAV1 and SAV2 induced lesions in infected fish

were more quantitative rather than qualitative (Boucher and Baudin Laurencin, 1996b). Another common feature for infections with SAVs is the impaired swimming performance, which for SAV2-infected rainbow trout often is described as “sleeping behaviour”. Thus, the impact the different subtypes of SAV have on the infected hosts is very similar, and the differences between the diseases traditionally known as PD and SD seem to be related to the principal main hosts and their farming conditions, as well as their geographical origin; PD/SAV1 from salmon in seawater (British Isles and Norway) and SD/SAV2 from rainbow trout in freshwater (France).

In order to supply the traditional diagnostic criteria (clinical signs and histopathology) other confirmatory tests have been developed. Different virological assays involving cell-culture (usually CHSE-214) isolation of SAV from diseased fish can be used, but has traditionally been regarded as difficult to interpret because CPE is not always present or may be indistinct (Desvignes et al., 2002; **Paper II**; Nelson et al., 1995). To overcome the fact that CPE induced by SAV is not a reliable indicator of virus growth, immunostaining techniques using mAbs have been developed to detect the presence of virus in cell-cultures (Graham et al., 2003b; Jewhurst et al., 2004; Todd et al., 2001). Immunostaining using mAbs is also implemented in virus neutralization (VN) testing for detection of SAV neutralizing Abs in fish serum (Graham et al., 2003a). It should be stressed that although VN often is regarded as the gold standard for antibody detection, a positive VN test does not necessarily confirm the presence of the virus itself. Furthermore, in the acute phase of a SAV infection, before the fish sero-converts (< 10 days post infection (McLoughlin et al., 1996)), a VN test would be negative.

Villoing et al. (2000b) presented a two-step RT-PCR assay for detection of SAV2 RNA in naturally infected salmonids, which also proved useful for amplification of SAV1 in experimentally infected fish. A similar RT-PCR technique has also been used to detect SAV3

RNA from Norwegian salmon (**Paper I**; Nylund et al., 2003b). However, these RT-PCR protocols cannot discriminate between the SAV subtypes without further sequencing studies. Recently, real-time RT-PCR protocols using TaqMan® MGB probes have been developed for SAV which greatly improves the sensitivity and specificity of the standard RT-PCR, and makes it is possible to differentiate between subtypes of SAV (**Paper III**). A less specific real-time RT-PCR assay using SYBR Green for detection of SAV in fish sera and tissues was later published by Graham et al. (2006). The increased specificity in a TaqMan probe assay compared to SYBR Green is a result of the different principles of detection. The dual-labelled TaqMan® probe is a single-stranded oligonucleotide that is complementary to a sequence within the target template (Figure 6), whereas the SYBR Green dye binds to any double-stranded DNA and is thus a sequence independent process.

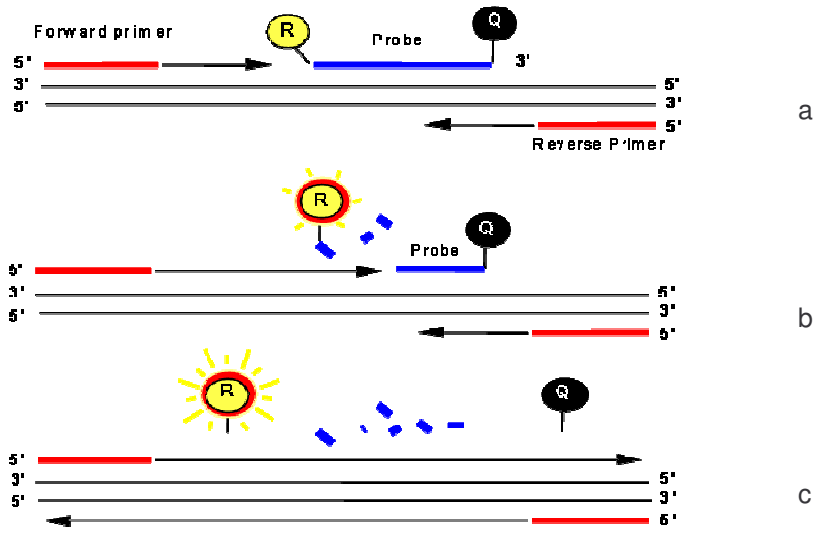

Figure 6. The TaqMan® probe is a sequence-specific probe that contains a fluorescent reporter dye (R) attached to the 5' end and a nonfluorescent quencher moiety coupled to the 3' end (Q). a) Before the probe is cleaved by the *Taq* polymerase the quencher fluorophore reduces the fluorescence from the reporter fluorophore. b) After annealing of the Taqman® probe the *Taq* polymerase start to add nucleotides and removes the probe from the template DNA. c) This separates the quencher from the reporter and allows the reporter to emit detectable light.

Fish sera; neutralising Abs against SAV

An immunological response in salmon to infections with SAV was first suspected by McVicar (1987) who noticed that surviving fish from outbreaks of SAV were protected against subsequent infections of SAV. Experimentally, the first antisera to SAV were raised in salmon following infection with SAV-infected kidney homogenate (Houghton and Ellis, 1996). Passive immunization with these sera was found to give up to 100% neutralisation with no pathology developing in the challenged fish, and it was concluded that the protection to SAV was a result of the fish producing neutralising antibodies (Abs). McLoughlin et al. (1996) performed a virus neutralising (VN) test by incubating sera with 200 $TCID_{50}$ virus (SAV1) for 2h before inoculating into CHSE-214 cells with subsequent CPE readings. Neutralising Abs to SAV were first detected in experimentally i.p. infected salmon as early as 10 dpi, while the Ab production in cohabitants was detectable 11 days after (12-15C). Based on the above results and the study by Desvignes et al. (2002) the majority of fish would be expected to seroconvert 3-6 weeks post-exposure at temperatures 12-15C.

Neutralising Abs was also detected in salmon from field outbreaks of SAV3 in Norway (Christie et al., 1998), and it was shown that these field sera reacted with the reference Irish virus isolate F93-125 (i.e SAV1) (Nelson et al., 1995). This serological cross-reaction between sera from Norwegian salmon and an Irish virus isolate was later confirmed by McLouglin et al. (1998), in a serological survey of the prevalence of neutralising antibodies to SAV in Irish, Scottish and Norwegian farmed Atlantic salmon. Experimental infection with SAV1 and SAV2 in both trout and salmon demonstrated the production of neutralizing Abs, and indicated full cross-neutralization (Weston et al., 2002). Serological cross-reaction with SAV1 was also detected in sera from SAV2-infected rainbow trout using an improved VN-test for Ab detection (Graham et al., 2003a). Here, an immunoperoxidase (IPX) based immunostaining using a monoclonal antibody (mAb) was developed for the detection of virus

growth in CHSE-214 cells, and was compared to the CPE-based VN detection in the original assay by McLoughlin et al (1998). Applying the IPX-VN assay on 353 farmed salmon and trout sera resulted in an overall seroprevalence of 25.7%, whereas all 188 sera collected from wild salmonids in freshwater localities in Northern Ireland were negative.

Polyclonal antisera and mAbs against SAV

The polyclonal mouse sera M4 was raised by Rowley et al (1998) and used to stain SAV1 infected CHSE-214 cells in combination with a biotin goat antimouse conjugate immunoperoxidase assay. Villoing et al. (2000a) produced a rabbit polyclonal antisera directed against a recombinant E2-protein from SAV2. When used in immunodetection of concentrated SAV2 virions it detected a single protein band of approximate molecular size of 47.5 kDa (Todd et al., 2001; Villoing et al., 2000a). This polyclonal E2 antiserum was later used in immunohistochemistry assays of infected pancreas, heart, muscle and brain with limited success compared to the RT-PCR protocol applied (Villoing et al., 2000b).

Monoclonal antibodies (mAbs) are currently utilized in many diagnostic procedures and are important tools in studies of pathogenesis. The first SAV specific mAbs were raised against whole virus of the Irish isolate F93-125; two mouse anti-SPDV monoclonal Abs (2D9 and 5D3) were produced and initially applied to infected CHSE-214 cells in combination with an immunoperoxidase detection assay (Rowley et al., 1998). These two mAbs, and the additional 1A9 mAb, also raised against F93-125, were used in a more comprehensive study by Welsh et al. (2000). They used the above three mAbs in various assays (indirect immunofluorescense (IIF) tests, RIPA with subsequent SDS-PAGE, immunodot blot), and demonstrated that 2D9 and 5D3 reacted with a single virus protein with a molecular mass in the 50-55 kDa range (Table 5). Based on the sizes of E1 (55 kDa) and E2 (50 kDa) analyzed by SDS-PAGE they concluded that 2D9 and 5D3 are reactive with an epitope of one of the two structural proteins.

Table 5. Monoclonal antibodies (mAbs) raised against SAV, with reference to their origin and application. Positive identifications of SAV isolates in the different applications are indicated in boldface, and negative identifications are underlined. The cited references are given as numbers for convenience, and corresponds to the numbering system of publications in the reference list.

			Application												
	mAb raised against:		IAP	IIF		Western Blot				RIPA		Other	Reference	
mAb	SAV subtype (isolate)	Protein-domain	SAV subtype (isolate)		cell location	Infiserte celler	Virus	*E. coli*	Putative protein	SAV subtype (isolate)	Putative protein		Publication	First published
1A9	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125)**	NC margin & cytoplasm					SAV1 (F93-125)	–	**SAV1 (F93-125)[1]**	141	141
2D9	SAV1 (F93-125)	Whole virus		**SAV1 (F97-12)**	NC margin & cytoplasm								112	112
	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125)**	NC margin					**SAV1 (F93-125)**	E1 or E2 (50-55kDa)	**SAV1 (F93-125)[1]**	141	112
	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**	NC margin & cytoplasm	SAV			–			**SAV[2]**	130	112
	SAV1 (F93-125)	Whole virus	**SAV1 (P42P), SAV2 (S49P)**	**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**									142	112
	SAV1 (F93-125)	Whole virus		**SAV2 (Scotland)**									45	112
	SAV1 (F93-125)	Whole virus		**SAV1, SAV2**	*								46	112
	SAV1 (F93-125)	Whole virus		**SAV1, SAV2**	*								59	112
	SAV1 (F93-125)	Whole virus		**SAV1**	*								43	112
4H1	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**	cytoplasm	**SAV**			E1 (53 kDa)				130	130
	SAV1 (F93-125)	Whole virus	**SAV1 (P42P), SAV2 (S49P)**	**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**									142	130
				SAV1, SAV2	*								59	130
5A5	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**	NC margin & cytoplasm	**SAV**			Capsid (35 kDa and 30 kDa)				130	130
	SAV1 (F93-125)	Whole virus	**SAV1 (P42P), SAV2 (S49P)**	**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**									142	130
				SAV1, SAV2	*								59	130
5D1	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**	cytoplasm	SAV			–				130	130
	SAV1 (F93-125)	Whole virus	**SAV1 (P42P), SAV2 (S49P)**	**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**									142	130
				SAV1, SAV2	*								59	130
5D3	SAV1 (F93-125)	Whole virus		**SAV1 (F97-12)**	NC margin								112	112
	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125)**	NC margin					**SAV1 (F93-125)**	E1 or E2 (50-55kDa)	**SAV1 (F93-125)[1]**	141	112
	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**	NC margin & cytoplasm	SAV			–				130	112
	SAV1 (F93-125)	Whole virus	**SAV1 (P42P), SAV2 (S49P)**	**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**									142	112
				SAV1, SAV2	*								59	112

(Continues on next page)

Table 5. Continued

7A2	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**	cytoplasm	SAV		–			**SAV[2]**	130	130
	SAV1 (F93-125)	Whole virus		**SAV2 (S49P)**								5	130
	SAV1 (F93-125)	Whole virus	**SAV1 (P42P), SAV2 (S49P)**	**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**								142	130
				SAV1, SAV2	*							59	130
7B2	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**	NC margin & cytoplasm	**SAV**		Capsid (35 kDa and 30 kDa)				130	130
	SAV1 (F93-125)	Whole virus	**SAV1 (P42P), SAV2 (S49P)**	**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**								142	130
				SAV1, SAV2	*							59	130
7C12	SAV1 (F93-125)	Whole virus		**SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)**	cytoplasm	SAV		–				130	130
I16	SAV2 (S49P)	Whole virus	SAV1 (P42P), **SAV2 (S49P)**	SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)								142	142
				SAV1, SAV2[†]	*							59	142
K16	SAV2 (S49P)	Whole virus	**SAV1 (P42P), SAV2 (S49P)**	SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)								142	142
				SAV2 (S49P)	*							59	142
L2	SAV2 (S49P)	Whole virus	SAV1 (P42P), **SAV2 (S49P)**	SAV1 (F93-125, F97-12, N2P6), SAV3 (N3P12)								142	142
				SAV1, SAV2	*							59	142
17H23**	SAV2 (S49P)	E2		**SAV1, SAV2**	at cellular membrane			E2		E2		89	89
19F3	SAV2 (S49P)	nsP1		**SAV1, SAV2**	cytoplasma, punctate	**SAV1, SAV2**	SAV1, SAV2	nsP1	**SAV1, SAV2**	nsP1		89	89
3E17	SAV2 (S49P)	nsP3		SAV1, **SAV2**	irregular spots in cytoplasma	SAV1, SAV2	SAV1, SAV2	SAV1, **SAV2** nsP3	**SAV1, SAV2**	nsP3		89	89
40D20**	SAV2 (S49P)	unknown		SAV1, **SAV2**	at cellular membrane							89	89
49K16	SAV2 (S49P)	E2		SAV1, **SAV2**	at cellular membrane	SAV1, SAV2	SAV1, **SAV2**	E2	SAV1, **SAV2**	E2		89	89
8A16	SAV2 (S49P)	nsP1		**SAV1, SAV2**	cytoplasma, dispersed	**SAV1, SAV2**	SAV1, SAV2	SAV1, **SAV2** nsP1	**SAV1, SAV2**	nsP1		89	89
71L2	SAV2 (S49P)	nsP1		**SAV1, SAV2**		**SAV1, SAV2**	SAV1, SAV2	nsP1	**SAV1, SAV2**	nsP1		89	89
78K5	SAV2 (S49P)	E1		**SAV1, SAV2**	cytoplasma, more intense close to nucleus	SAV1, **SAV2**	SAV1, **SAV2**	SAV1, **SAV2** E1	**SAV1, SAV2**	E1		89	89
4I16	SAV2 (S49P)	E2		SAV1, **SAV2**	at cellular membrane	SAV1, SAV2	SAV1, **SAV2**	E2	SAV1, **SAV2**	E2		89	89
51B8	SAV2 (S49P)	E2		**SAV1, SAV2**	cytoplasma, dispersed		SAV1, **SAV2**	SAV1, **SAV2** E2	SAV1, SAV2	E2		89	89

* Immunoperoxidase-based neutralization assay (IPX-VN)

** Neutralizing mAbs

[†] Positive fluorescence for reference strain S49P only

[1] Dot Blot

[2] ELISA

Later, several additional mAbs have been raised against SAV1 and SAV2 whole virus (Moriette et al., 2005; Todd et al., 2001; Weston et al., 2002) and tested for reactivities against different subtypes of SAV. In general, the above mAbs show extensive crossreactivities with all tested subtypes of SAV (Table 5). Possible exceptions are the three mAbs 40D20, 4I16/I16 and 49K16/K16, all raised against the SAV2 isolate S49P, and which only reacts with SAV2 in IIF- and RIPA (Jewhurst et al., 2004; Moriette et al., 2005; Weston et al., 2002). However, in these studies positive staining reaction using the three mAbs was only observed with the reference SAV2 isolate, and not when field isolates of SAV2 were tested (Jewhurst et al., 2004; Weston et al., 2002).

Moriette et al. (2005) also mapped and characterized six additional mAbs raised against recombinant *E. coli*-expressed SAV2 proteins. These mAbs were directed against nsP1 (8A16, 19F3 and 71L2), nsP3 (3E17), E2 (51B8) and E1 (78K5), where the 3E17 mAb was able to discriminate between SAV1 and SAV2 in an IIF assay. Localization of some SDV proteins was suggested based on the staining pattern in infected cells for the different mAbs. Two of the nsP1-derived mAbs, together with the nsP3-derived mAb, showed positive reaction associated with type I cytoplasmic vacuoles (CPVIs). The staining pattern for the third nsP1-derived mAb was more dispersed in the cytoplasm. The SAV2 E2 protein was recognized either in the cytoplasm (51B8) or at the cellular membrane (4I16, 79K16 and 17H23), whereas the E1 protein reacted with mAb 78K5 in the cytoplasm. The latter mAb was also used for positive detection in an immunohistochemistry (IHC) assay from SAV2 infected red muscle and pancreas (Moriette et al., 2005).

While sera from survivors of previously SAV-infected fish have neutralizing Abs, it has been difficult to show any virus neutralizing activity from available SAV mAbs (Moriette et al., 2005; Todd et al., 2001). However, in Moriette et al (2005) neutralizing properties were

demonstrated from two mAbs (17H23 and 40D20), both directed against whole virus of the SAV2 type isolate S49P.

Epizootiology

Disease outbreaks caused by SAV1 and SAV3 are reported mainly between March-November, but can occur throughout the year. On average it takes 2-3 months post transfer to sea for SAV1 to infect a farm site in the British Isles (McLoughlin et al., 2002), but in Norway the average period from sea transfer to outbreak of SAV3 is 7-9 months (E. Brun, National Veterinary Institute, Norway, pers. comm.). However, outbreaks of SAV can occur as early as a 5-8 weeks after the smolt have been released into sea (Crockford et al., 1999). The duration of a SAV outbreak in a farm site can vary substantially; from 1-4 months in Ireland (McLoughlin et al., 2002), to an average of 10 weeks for Norwegian sites (E. Brun, National Veterinary Institute, Norway, pers. comm.). The severity of an outbreak and associated mortalities also varies greatly from only a few percent to more than 40% (McLoughlin et al., 2002). In summary, the onset, duration and severity of a SAV1&3 outbreak show considerable variation, and there are indications suggesting that this is related to water temperature, environmental factors such as feeding regime, smolt strain and regional differences. Outbreaks of SAV2 in rainbow trout in freshwater localities can be observed in fish of any age, but generally affect fish from 10-50g in spring when the water temperature is between 9-13°C (Boucher and Baudin Laurencin, 1996a). The mortalities associated with SAV2 are relatively low (5%), but as for SAV1 and SAV3 it may vary from a few percent to over 40% in some cases. A typical outbreak with SAV2 lasts for approximately 2 months.

Indications that surviving fish from outbreaks of SAV develop a long-term protection to new infections was first noted by McVicar (1987), and this was later verified in experimental infection trials for both SAV1 and SAV2 (Boucher and Baudin Laurencin, 1996b; Houghton,

1994). Furthermore, acquired cross-protection against SAV1 and SAV2 was observed in *O. mykiss* after experimental infection with either SAV1 or SAV2 (Boucher and Baudin Laurencin, 1996b).

The subtypes of SAV are principally located to three different enzootic areas; SAV1 in British Isles, SAV2 in France and SAV3 in Norway (Figure 7). There are, however, some exceptions which complicate this clear-cut distribution. Recently, SAV2 has been found in Scotland and England (Branson, 2002; Graham et al., 2003b), but these incidents are considered a results of the import of SAV2 infected fish from France (Weston et al., 2005). SAV2 has also been isolated from diseased rainbow trout from Germany on one occasion, and is suspected, but not confirmed, in freshwater fish in Italy (Boscher et al., 2006). Unfortunately, no further information on virus and host origin is presently available. Kent and Elston (1987) described a disease condition compatible with SAV infection in pen reared Atlantic salmon in North America, but the true origin of the virus in this case-report can be questioned (see General discussion for details). Years later, Kibenge et al. (2000) reported a novel togavirus-like virus from salmon in New Brunswick (Canada), and were able to isolate and grow the virus in cell-cultures. A 330 bp PCR-amplicon from the togavirus-like virus cDNA was produced, but no nucleotide sequence is presently available for further identification of this virus. Since the virus-isolate was non-pathogenic in experimental infected salmon, it seems less likely that the togavirus-like virus can be assigned to SAV. Locally in Norway, SAV3 is now reported in new areas separated by more than 1000km from the enzootic focus in western Norway (Figure 8). However, these disease outbreaks with SAV3 in northern Norway can be traced back to transport of smolts from the enzootic area (**Paper II**), and as such is a clear parallel to the SAV2 incidences in Scotland and England (Graham et al., 2003b; Weston et al., 2005).

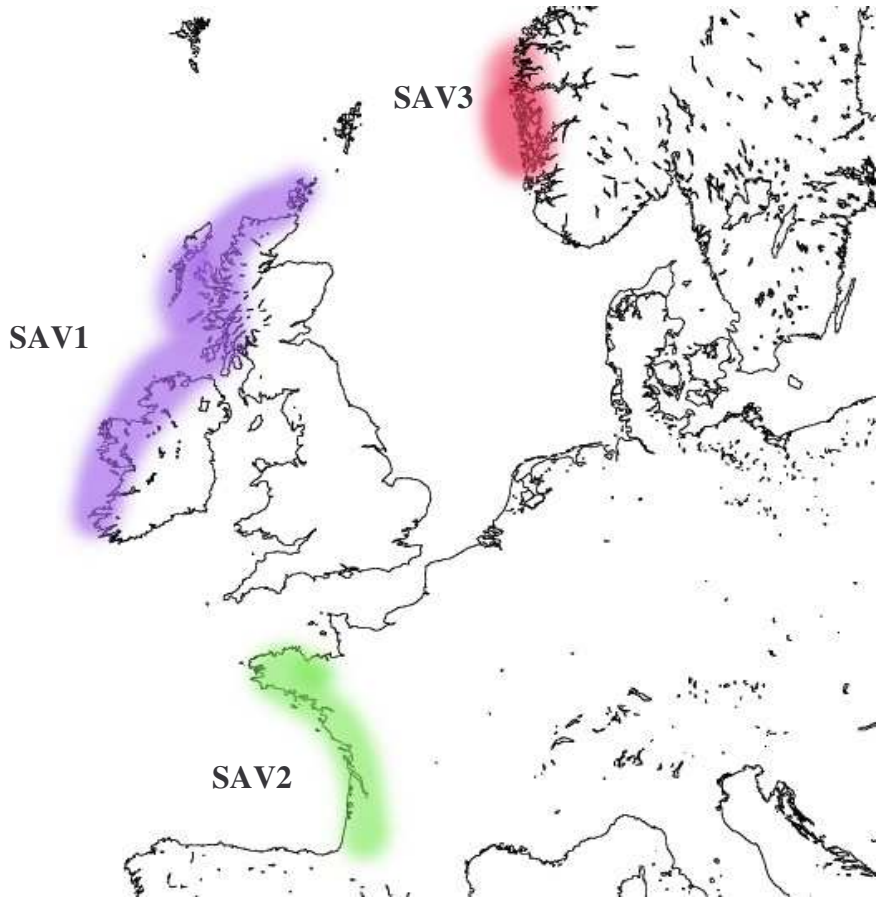

Figure 7. The subtypes of SAV are principally located to three different enzootic areas; SAV1 in British Isles, SAV2 in France and SAV3 in Norway

Vertical and horizontal transmission of SAV?

Successful transmission trials with SAV have been performed numerous times, mainly through intraperitoneal injections but also in cohabitant studies, thus demonstrating that horizontal infections with SAV can occur. Circumstantial evidence from salmon farms also points in the direction of horizontal spread of the virus between cages within single fish farms. In a artificial environment such as in fish farms the stocking densities can be very high, and horizontal spread of the virus within a single cage and between cages will be effective. However, the significance of horizontal spread of SAV between farm localities or regions is not clear. Here, transport of infected fish in well-boats seems to better explain the

observations of new SAV outbreaks in localities previously free of the virus (**Paper II**; E. Brun, National Veterinary Institute, Norway, pers. comm.).

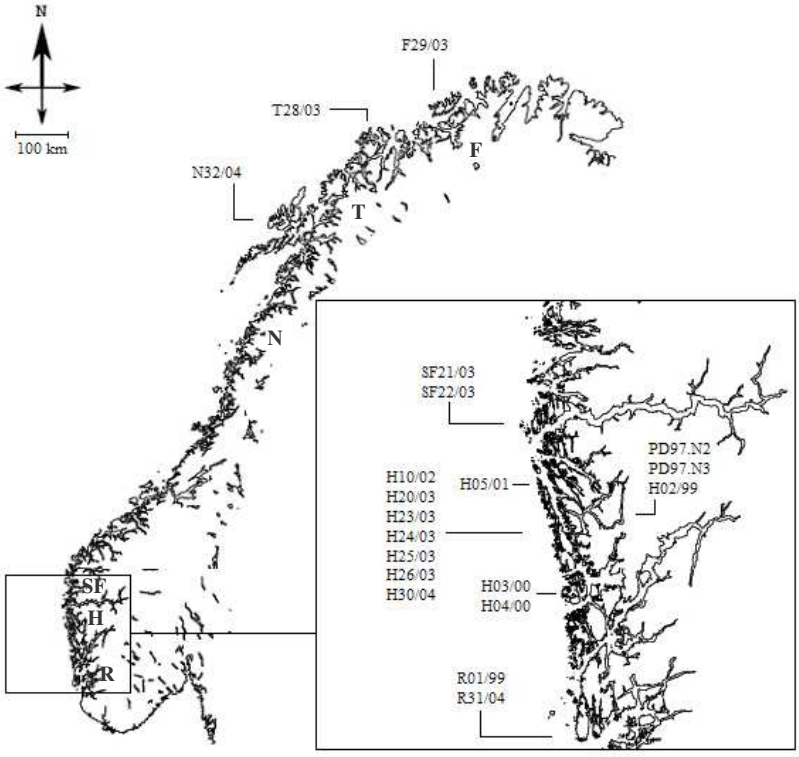

Figure 8. The coastline of Norway. Locations where the SAV3 isolates were collected are indicated. The isolate prefix (SAV-) is left out for convenience. See **Paper II** for details on the different SAV isolates. Counties that are discussed in the text are indicated on the map as follows: F=Finmark, T=Troms, N=Nordland, SF=Sogn og Fjordane, H=Hordaland, R= Rogaland

The fact that Atlantic salmon populations from the entire northern Europe intermingle in their feeding grounds in the north Atlantic (Hansen and Jacobsen, 2003; Jacobsen and Hansen, 2001) should favour a possible horizontal spread of SAV subtypes. However, the absence of such crossinfections of SAV-subtypes suggests that horizontal spread in the wild is inefficient without involving a common vector, or situations where fish come in closer contact with each other. Closer contact between fish is obtained in a farming environment or in rivers in connection with spawning. Alternatively, it is possible that there are differences in susceptibility to infections with the SAV-subtypes in wild salmon from British Isles and Norway which prevent any crossinfections, although this seems less likely.

The question of whether vertical transmission of SAV is possible remains unclear. Earlier it was argued by several authors that horizontal spread was the only transmission route for SAV, but recently Castric et al. (2005) were able to re-isolate SAV2 from batches of egg and the 2 months old progeny from the experimentally infected broodfish. Similarly, it has been shown that SAV3 can be detected (by real time RT-PCR) in eggs and fry originating from naturally infected broodfish (A. Nylund, University of Bergen, Norway, pers. comm.). Vertically transmitted virus could also be the origin for the outbreak of SAV in salmon reported from North-America by Kent and Elston (1987) (see General discussion, page 47). If vertical transmission of SAV is a reality, it is quite possible that once SAV is introduced into the fish farming system a virus-isolate will be self-sustained due to the unnaturally high densities of susceptible hosts during all phases of the production cycle compared to the natural environment.

Reservoir for SAV?

Infection and transmission of SAV1 and SAV3 have through history been considered to occur in seawater, yet no good evidence exists for a marine reservoir. Circumstantial evidence has been presented by among others McVicar (1987), who mention numerous examples of a single smolt unit in Scotland providing fish which subsequently become affected in some sea farms but not in others. He also noted that there is a tendency that once a farm has become affected the disease re-appears with the yearly intake of new smolt. A marine origin of SAV is also argued in a study by Ferguson et al. (1986a) where fish from the same hatchery were transferred to two sites, but only developed the disease in the site where the disease was enzootic.

However, there are indications of both a freshwater reservoir and freshwater transmission for the virus subtypes. Firstly, SAV3 has been detected from freshwater smolts prior to the

transfer to sea (Nylund et al., 2003b, pers. obs.), which indicates either a vertical transmission or infection in the freshwater phase. Secondly, the geographically delimited disease problems in western Norway caused by SAV3 seem to be caused by a very homogenous virus reservoir. In the study on the molecular evolution of SAV3 a striking genetic homogeneity within the Norwegian sequence isolates was demonstrated, although the isolates covered a relatively large geographical area over a period of eight years (**Paper II**) (Figure 8). This suggests that some isolating factors exist that prevents further dissemination to new areas outside western Norway. As mentioned earlier the recent examples of SAV3 in northern Norway are believed to be a result of transport of SAV3 infected fish from smolt producers located in the enzootic region (**Paper II**), and hence should not be included in the natural geographical range of SAV3. For SAV3, the observed genetic homogeneity within the well defined enzootic focus is most likely explained by either extensive geneflow within the virus reservoir, or a common source of virus. A possible scenario with freshwater transmission and maintenance of SAV3 should therefore not be ruled out. In this phase of their lifecycle Atlantic salmon occur in much higher densities in the river systems than is the case in the marine phase, and would make in-contact (horizontal) virus transmission more probable. Whether such freshwater transmission of SAV involves an arthropod host or not is unknown.

Finally, it should also be noted that the kind of strict geographical distribution of the three different SAV subtypes is also a common feature for terrestrial alphaviruses, and is believed to reflect isolated host populations and viral geneflow within them (Brault et al., 1999; Kramer and Fallah, 1999; Lindsay et al., 1993; Mackenzie et al., 1995; Oberste et al., 1999; Strauss and Strauss, 1994; Weaver et al., 2004; Weaver et al., 1997). An original strict distribution of genotypes within ISAV is also proposed, and can be explained by a maintenance of virus in wild populations through local freshwater transmissions (Nylund et al., 2003a). However, a recent study on genotyping of ISAV isolates strongly suggests that

transport of ISAV infected salmon has resulted in a more widespread distribution of ISAV genotypes in Norway (Nylund et al., 2006). Fish viruses utilizing marine reservoirs such as VHSV and IPNV have generally widespread genotypes that are not restricted to enzootic foci (Benmansour et al., 1997; Einer-Jensen et al., 2004; Snow et al., 2004; Thiery et al., 2002; Zhang and Suzuki, 2004), and absence of such distinction reduces the probability of a marine reservoir for SAV.

Vector transmission of SAV?

An important issue concerning the dissemination of SAV involves the possible involvement of an arthropod vector, a common feature for other alphaviruses. Terrestrial alphaviruses, whose main host is often a bird or mammal, are arthropod borne (arbo-) viruses that are transmitted by an insect vector in which they are able to replicate. To date, the only known alphaviruses associated with the marine environment are SAV and the recently characterized alphavirus isolated from a parasitic louse (*Lepidophtirius macrorhini*) on the southern elephant seal (Linn et al., 2001). Although *L. macrorhini* is strictly a terrestrial arthropod and there is no conclusive evidence that it represents a true vector of the seal alphavirus, the results have fuelled speculations originally put forward by Weston et al. (1999) that an arthropod vector could be involved in the transmission of SAV in salmon. Obvious arthropod candidates for possible transmission of SAV1 and SAV3 would be the salmon louse *Lepeophtheirus salmonis* and/or *Caligus elongatus*, which are common ectoparasites on wild and farmed salmon in seawater. Indeed, SAV3 has been detected from *L. salmonis* by real-time RT-PCR collected from diseased fish (M. Karlsen, University of Bergen, Norway, pers. comm.), but it is not clear whether the source of virus is infected blood meals or actively replicating virus in the salmon louse. If *L. salmonis* were a transmission vector for SAV1 &3 the distribution of the different SAV genotypes should reflect the distribution of the *L.*

salmonis populations. Lice burden can be considerable on salmon in the feeding grounds in the North Atlantic and circumstantial evidence indicate that *L. salmonis* infestation occurs in these areas (Jacobsen and Gaard, 1997), which is further supported by recent studies demonstrating a lack of genetic differentiation of the North Atlantic *L. salmonis* populations (Tjensvoll et al., 2006). In contrast, the SAV subtypes have a geographically island like distribution with SAV1 in the British Isles, SAV2 in France, and SAV3 exclusively in Norway. Thus, no spread of subtypes occurs between these different geographical host populations, which in turn make the involvement of *L. salmonis* as a transmission vector for SAV unlikely.

Potential use of SAV in vaccinology

An advantage of the alphaviruses is that the RNA genome itself is infective to a host cell. This feature has been exploited by recombinant DNA technique where an infective full-length cDNA clone of the viral genome is synthesized. By conversion of the RNA genome into complementary DNA (cDNA), an intermediate can be generated that is amenable to genetic modification and which can subsequently be converted back – either *in vitro* or *in vivo* - into an RNA genome which is able to yield infectious virus. This process is known as 'reverse genetics'. The positive-stranded RNA viruses, such as SAV, can be directly used for translation by the host cell machinery and initiate an infectious cycle. In the classical RNA-launched approach, cells are transfected with RNA transcripts made from the infectious cDNA clones, and the synthetic viruses are then recovered from these cells (Liljestrom et al., 1991; Rice et al., 1987). However, an alternative DNA-launched approach also exists that was first reported for poliovirus and has later been adapted for alphaviruses (Schlesinger and Dubensky, 1999). Here, synthetic viruses are generated by directly transfecting infectious cDNA clones into susceptible cells. Both of these approaches have been used to construct

infectious cDNA clones which have been invaluable in addressing many questions regarding the positive-sense RNA viruses. The cDNA full-length clone may be used as a vector for the expression of foreign proteins in eukaryotic cells, but it can also serve as a powerful tool in evaluating what effect substitutions, insertions or deletions have on virus replication (Frolov et al., 1996).

Recently, an infectious full-length cDNA clone of the Salmonid alphavirus (SAV2) was engineered by Moriette et al. (2006) in France. The 11,894 SAV2 genome was inserted into a transcription plasmid, pSDV, and effectively transfected into BF-2 cell-cultures with subsequent recovery of recombinant virus, rSDV. In vivo infectivity of rSDV in fish was confirmed by immersion of juvenile rainbow trout in a water bath with rSDV and two wild type virus isolates (wtSDV). All fish became infected with a virus titer of approx 10^7 PFU/ml for both rSDV and wtSDV. While the cumulative mortality after 60 days reached 78 % for wtSDV, no mortality was observed in the rSDV infected trout during the same period of time. The lack of pathogenicity of the rSDV was found to be associated with the temperature at which the viruses were produced; at 10°C rSDV was non-pathogenic to trout while it became pathogenic when grown at 14°C. The shift in the temperature was shown to be associated with the appearance of amino acid changes in the SDV structural proteins E2, 6K, and E1. In addition, when the rSDV-infected fish were challenged after 3 and 5 months with wtSDV and SAV1, no mortalities was observed. Thus, the protective properties of rSDV are promising for the application of the recombinant SDV as a potential vaccine against SAV. Also, the possibility for the rSDV to express foreign protein was explored. Here, a rSDV encoding heterologous protein representing more than 20% additional sequence was successfully transfected and expressed, which suggests that the established pSDV system also has potential as an effective expression vector in salmonids.

2 AIMS OF THE PRESENT STUDY

In Norway, at the time when the project started (2001), little was known about the virus responsible for the mortalities allegedly caused by the disease known as PD. Initially, an important goal was to develop more sensitive and specific diagnostic tools for this virus, since a clear-cut diagnosis often was precluded by similar clinical and histopathologtical lesions associated with other disease conditions. A strategy involving PCR detection of the viral genome was decided as the method of choice. It soon became evident that the virus from diseased fish in Norway was genetically different from SAV1 in the British Isles. Therefore, the project was extended to include a molecular characterisation of the Norwegian subtype of SAV.

Thus, the specific aims of the present study were to:

- Genetically characterise the Norwegian subtype of SAV.
- Develop a sensitive and specific test for the detection of SAV, and use this to
- Study the geographical distribution of SAVs in Norway.

3 OVERVIEW OF PAPERS

Paper I. This work presents the first molecular description of the virus responsible for pancreas disease (PD) in Norway. The virus was isolated from moribund fish suffering from pancreas disease (PD) from different locations in western Norway; one isolate originated from rainbow trout (*Oncorhynchus mykiss*), whereas the remaining three were collected from farmed salmon (*Salmo salar*). Based on analyses of the near full-length nucleotide sequence it is evident that these isolates represent a single entity, which is significantly different compared to the other members of the Salmonid Alphavirus (SAV). This contradicts the

previous hypothesis that PD in farmed salmonids in Norway is caused by the same virus as that described in the British Isles; Salmon Pancreas Disease Virus (SPDV). The new virus strain (SAV subtype 3) seems to be exclusive for PD isolates in Norway and is per date not located elsewhere.

Paper II. The classification of the alphavirus species SAV is now comprised of at least three subtypes 1-3; SAV1 isolated from salmon *Salmo salar* in Ireland and the British Isles, SAV2 isolated from rainbow trout in France and the British Isles, and new SAV3 described in **Paper I**. The present paper has investigated the phylogenetic relationships among 20 SAV3 isolates, based on a 1221-ntlong segment covering part of the capsid gene, E3, and part of the E2 gene, that were collected over a period of eight years. The isolates covered a large geographical area from Rogaland in the south to Finmark in northern Norway. All isolates were of the SAV3 subtype and supports the notion in **Paper I** that this is the only subtype of SAV occurring in Norway. Furthermore, the results revealed genetic homogeneity among SAV3 isolates and a low substitution rate suggesting that some mechanism(s) exist to stabilize the molecular evolution of SAV3. The genetic stability of SAV3 was also studied in CHSE-214 cells. Sequencing of the SAV3 genome (11530 nt) after 20 passages revealed only 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.

Paper III. The recent discovery that pancreas disease in Norway is in fact caused by a new and distinct subtype of salmonid alphavirus (**Paper I** and **II**), exclusively found in Norway, has advocated the need for better diagnostic tools. In the present paper, three real-time PCR assays for all known subtypes of salmonid alphavirus have been developed; the Q nsP1 assay is a broad-spectrum one that detects RNA from all subtypes, the Q SPDV assay specifically

detects the salmon pancreas disease virus subtype, and the Q_NSAV assay only detects the new Norwegian salmonid alphavirus subtype. The results demonstrated the assays to be highly sensitive and specific, detecting <0.1 TCID50 of virus stocks, and were reproducible over a wide range of RNA input. Thirty-nine field samples were tested in triplicate and compared with traditional RT-PCR. Overall, the real-time assays detected 35 positive compared to 29 positives in standard RT-PCR, and were thus shown to be more sensitive for detecting salmonid alphaviruses in field samples. The real-time PCR assays are excellent tools for monitoring or screening purposes, and have great potential in future quantitative studies of SAV.

4 GENERAL DISCUSSION

The aims of this thesis has been to supplement the present diagnostic tools for SAV and, by implementing the new and improved detection method, try to get a better overview and understanding of the distribution of SAV in Norway. In the course of this study it became evident that the Norwegian isolates of SAV were unique compared to the two subtypes of SAV previously described from Ireland and France. Hence, a genetic characterisation of the Norwegian SAV subtype was imperative and became a central part of the thesis.

Genomic sequence diversity within SAV

Analysis of the genomic sequences from the four Norwegian virus isolates in **Paper I** show that they possess a genome organization that is identical to that observed for the other two SAV isolates, SAV1 and SAV2 (Weston et al., 2002), and for mammalian Alphaviruses (Strauss and Strauss, 1994). In summary, the body of evidence from phylogenetic analysis based on the amino acid or nt sequence of different virus isolates available in the Genbank shows that these viruses constitute a distinct species (Salmonid Alphavirus, SAV) within the genus *Alphavirus* of the family *Togaviridae* (Weston et al., 2002; present study) (Figure 9). SAV can further be divided into the three subtypes SAV1, SAV2 and SAV3 (**Paper I**; Weston et al., 2002), and these subtypes seem to have distinct geographical distributions; SAV1 has a geographical basis in the British Isles, SAV2 originates from France but is now also present in the British Isles and Germany, and SAV3 which is enzootic to Norway (**Paper II**). A recent addition to the sequence variation within SAV was reported by Weston et al. (2005) in a sequence study on sequences of E1 and nsP4 gene fragments from SAV isolates originating from British Isles, France and Norway. Interestingly, they identified a variant of SAV1 isolated from salmon in Scotland (Western Isles), which showed consistent sequence differences from all the three subtypes of SAV, but was more closely

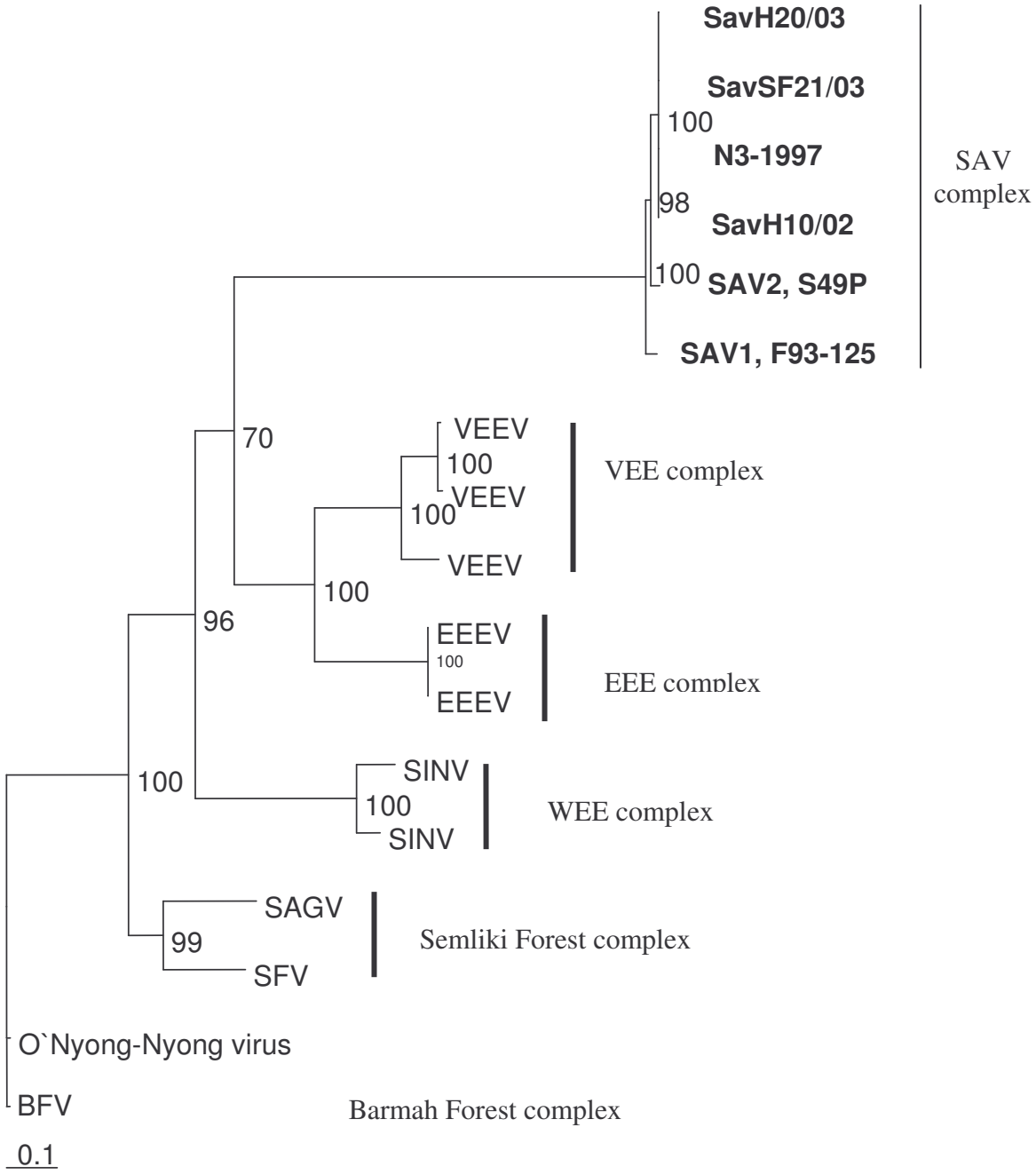

Figure 9. The phylogenetic position of the SAV3 isolates (SavH20/03, SavSF21/03, SavH10/02 and N3-1997) in relation to other salmonid Alphaviruses (SAV) and mammalian Alphaviruses. The evolutionary relationship is presented as a maximum likelihood tree based on alignment of non-structural polyproteins amino acid sequences from selected members of genus Alphavirus. The scale bar shows the number of amino acid substitutions as a proportion of the branch lengths. EEE=eastern equine encephalitis, VEE= Venezuelan equine encephalitis WEE=western equine encephalitis.

related to SAV1. The evolutionary significance of this variant of subtype 1 SAV is uncertain, and so far such intermediate sequence variants are not reported for SAV2 or SAV3. However, this clearly demonstrates the need for more sequence isolates of SAV in order to gain better insight into the molecular diversity of SAV. This is exemplified by the acknowledgement of the new Norwegian subtype of SAV (**Paper I**), a virus that has for different reasons been erroneously designated as SAV1, but is now regarded as the only subtype circulating in Norway.

A new addition to the sequence diversity of SAV may come from North America. If the disease condition reported from Atlantic salmon in Washington state by Kent and Elston (1987) is caused by SAV, and their assumptions that the virus is of local marine origin, it is quite possible that this could represent yet another subtype of SAV that is different from the European subtypes. However, it seems peculiar that this case-report by Kent and Elston (1987) is the sole record of infection of salmonids in North America, and has never subsequently been observed in this region hereafter. On the contrary, it seems more probable that the imported salmon eggs from Scotland and Norway, from which the diseased fish were hatched and reared from, is the source of the virus. If this were the case then the virus causing the disease condition in Kent and Elston (1987) could be assigned to SAV1 or SAV3. Not only would this be an example of the importance human activities have on the spread and distribution of diseases, but it also indicates that vertical transmission within SAV may occur. A novel togavirus-like virus has also been isolated from fish suffering from ISA in New Brunswick, Canada (Kibenge et al., 2000). However, the experimental infection trials and sequence data deposited in the Genbank are not compatible with a SAV aetiology.

Two other examples of introduction of SAV to new areas as a result of human activities are known; import of SAV2 infected rainbow trout from France to two sites in England and

Scotland (Branson, 2002), and transport of SAV3 from smolt producers in Western Norway to marine sites in Northern Norway (**Paper II**). Although not conclusive, the latter case study is believed to be a result of well-boat transport of SAV3 infected smolts to locations with no prior history of SAV. This is supported by the observation that the genotype sequence from these recent outbreaks in northern Norway is very similar or identical to the isolates from Western Norway at least 1000 km to the south (**Paper II**).

RNA viruses commonly have substitution rates in the range of 10^{-3} to 10^{-5} misincorporations per nt copied (Drake and Holland, 1999), which for SAV should result on average at least one substitution introduced per genome replication. Although the SAV3 isolates herein is diverse with respect of time and space, the sequences show surprisingly little variation as demonstrated in the calculated evolutionary rate of $1.7x10^{-4}$ nt substitutions/site/year. As previously speculated this could be a result of stabilizing selection often seen in arboviruses that replicate in two alternating hosts, and could therefore indicate the presence of a vector for SAV3. However, it was recently shown that ISAV, a salmonid virus without vector replication, has even lower evolutionary rates than SAV3 (Devold et al., 2006; Nylund et al., 2006), a result that may question the hypothesis of a vector transmission for SAV3.

The low evolutionary rates for SAV3 only reflects the sequence data used in the analysis, and including gene fragments other than these used in the present study (capsid, E2 and E3) may result in different evolutionary rates. It is also possible that the low evolutionary rates for SAV3 is an artefact resulting from selective sampling, since all our sequence isolates are collected from a relatively homogenous host population (i.e farmed salmonids in seawater). This biased sampling also applies to the other SAV subtypes; to date, there is no record of SAV from wild fish and all isolates and sequences originate from farmed salmonids. Thus, the question arises whether the existing sequence diversity of SAV represents the true diversity, or perhaps only mirrors the recycling of a few selected isolates that is maintained and

multiplied in the farming industry in the respective countries? If the sequence diversity reported from Norwegian SAV isolates is the true diversity for SAV3, then this could indicate a recent introduction of SAV to Norway. An introduction of SAV (for instance through import of salmonids from other countries) could result in an establishment of a new SAV population carrying only a small fraction of the variation from the original SAV population (quasispecies). The resulting genetic diversity from such a genetic bottleneck will be low, since the founder population only consists of a limited number of virus individuals (Manrubia et al., 2005).

Real time PCR as a screening- and diagnostic tool

The success in obtaining wild–type isolates of SAV is very much dependent on the sensitivity of the screening tools, locality, life history stage of the fish host, prevalence and amount of virus in infected tissue, tissue tropism and number of samples examined. Any new field isolates from a non-farming site would also give important indications on natural SAV reservoirs. Some effort has been made to screen wild population of salmon and trout for SAV using the IPX-based VN-assay described by Graham et al. (2003a). Sera from approximately 400 wild salmonids from Ireland and 42 from Norway have been tested, but none were positive (Graham, 2005; Graham et al., 2003a). This could imply that wild fish cannot be considered an important reservoir of SAV infection for farmed populations. It is, however, a possibility that serological testing is not sufficiently sensitive to detect subclinical or latent carriers of SAV. Moreover, the occurrence of SAV in wild fish may very well be delimited/defined in time and space, which makes it crucial to know when and where to sample fish to increase the likelihood of retrieving new wild-type isolates of SAV. Since we do not know any details on SAV in wild fish this can only be speculated on, but any

information regarding the anamnesis of outbreaks of SAV may indicate or be decisive for a sampling strategy.

A sensitive tool is needed when screening subclinical amounts of SAV. The TaqMan based real-time RT-PCR protocol developed for detecting RNA from all subtypes of SAV should prove useful (**Paper III**). It was demonstrated that the Q_nsP1 assay was 10-100 fold more sensitive than standard RT-PCR on all virus stocks tested, and when applying this test on field samples the number of positive fish increased by 15% compared to standard RT-PCR. How the Q-nsP1 assay performs compared to the IPX-VN assay applied in the literature (Graham, 2005; Graham et al., 2002; Graham et al., 2003a) is not known, but the relationship between presence of neutralizing Abs and Ct-values (i.e amount of viral RNA) from real-time RT-PCR assays should be examined in detail. Ideally, a combination of serology and real-time RT-PCR testing should be performed for each fish sampled, which should provide more detailed information on the viraemic stage of the fish. If both the IPX-VN and Q_nsP1 assays were negative in a combined test then it is highly probable that the sample is negative for SAV. When both IPX-VN and Q_nsP1 are positive then a current infection with SAV is present. A situation where the IPX-VN is negative but with low Q-nsP1 values also represent a current infection, but is in an early stage of infection before the fish seroconverts (approx. two weeks). Last, if the IPX-VN is positive and Q-nsP1 is negative this would represent either a previous infection where the hosts immune response (neutralizing Abs) have cleared the virus, or a persistent or latent infection in carrier fish with low, undetectable virus numbers. The increased sensitivity (and specificity) of the Q_nsP1 assay may prove particularly useful for addressing the important question whether SAV can exist as a latent or persistent infection in the host. Persistent infection of SAV has been demonstrated in experimental infection trials in salmon (unpubl. results) and is discussed in more detail below.

Diagnosis of diseases caused by SAV (PD and SD) has previously, and is still mainly based on clinical signs in combination with histopathological findings. Also, detection of specific antibodies in fish sera (see above) and virus isolation in cell cultures have been applied to verify the aetiology (Graham et al., 2003a; Jewhurst et al., 2004), but none of these methods are capable of discriminating between the different SAV subtypes. Moreover, the presence of virus-specific antibodies in serum only states that the fish has been exposed to SAV minimum 10-14 days prior to sampling, and thus provide only limited information on the viraemic status of the fish. Presently, there is a large panel of mAbs for direct detection of SAV antigens (Table 5) but since most of them show extensive cross-reactivities with the other subtypes this probably makes them unsuitable for distinguishing subtypes of SAV. However, some of the mAbs capable of reacting with all three subtypes of SAV should have the potential for a diagnostic test common for all SAV. To overcome the fact that the prevailing diagnostic methods were not sufficient to rapidly distinguish between the different pheno-/genotypes, standard RT-PCR based protocols were developed for SAV (**Paper I**; Nylund et al., 2003b; Villoing et al., 2000b). Combined with sequencing of the RT-PCR amplicons this greatly enhanced the sensitivity and specificity of detecting SAV in tissues. Further refinement of this methodology is presented in **Paper III** where a TaqMan based real-time RT-PCR protocol is described in detail. To date, this method represents the most sensitive, specific, labour- and time saving method for identification of viral RNA of any SAV subtype, and has a great potential as a diagnostic tool in fish medicine. It can also be used to estimate the viral RNA load in any tissue, either as absolute quantification or relative quantification. In most cases it is sufficient to merely document the relative changes of SAV-templates between varying experimental conditions (Bustin, 2000; Mackay et al., 2002; Pfaffl, 2001). This can be achieved by simultaneously monitoring a non-regulated reference target; either internal or externally added.

As for all diagnostic tests the type of tissue samples used will influence/have great impact on the test results, and it is of the utmost importance to have some knowledge of the tissue and organ distribution of the pathogen in question. To address this issue, the newly developed real-time RT-PCR assays for SAV are ideal to monitor and relative quantify viral RNA from different tissues in salmon during outbreaks of SAV. In an experimental infection of salmon with either SAV1 or SAV3 it was possible to detect viral RNA in fish tissues at all stages of the disease, including previremia, for as long as 190 days after i.p injection (Andersen et al., submitted). The infected fish showed no clinical signs of disease during this viral persistence, which indicated that surviving salmon became asymptomatic carriers of SAV. The temporal relative changes in SAV load in the experimentally infected salmon were normalized to both an internal control gene (Elf-1, (Olsvik et al., 2005)) and external added control (Influenza A RNA). The results from the Q_nsP1 assay also show that the pseudobranch and heart tissue (ventricle) were best suited for diagnostic purposes regardless of disease status. On the other hand, pancreatic tissue was considered unsuitable for real-time RT-PCR testing of SAV RNA. Moreover, the observation of a high percentage of SAV positive gill tissues in the experimentally infected fish should enable non-lethal gill biopsies in future screening of valuable broodstock fish and/or wild salmonid stocks.

As pointed out in **Paper II** the reliability of cell culture detection of SAV is hampered by the fact that virus-induced CPE is weak or not always present. This problem can be overcome by simply using real-time RT PCR detection and quantification of SAV, making the subjective interpretation of the presence/absence of CPE in the cell-culture redundant in terms of virus replication. A protocol for the relative quantification of SAV in CHSE-214 and ASK cells using the Q_nsP1 assay has now been developed (unpubl. data), where the Q_nsP1 Ct-values from a fixed number of cells are normalized to the internal Elf-1 Ct-values and an externally added reference gene. The initial 10^2-10^3 fold increase in virus production in the cell-cultures

(ASK and CHSE-214) stabilizes after 48 hours, and remains constant thereafter until a slight decrease is observed between 192 and 336 hours after infection. Parallel to the monitoring of virus production with time, the normalized expression profiles of the type I interferon α (IFN-α) and the GTP-ase Mx protein were recorded. In both ASK and CHSE-214 cells IFN-α and Mx were upregulated, with a slight lag for the Mx response. Interestingly, the upregulation of IFN-α/Mx was significantly higher in the SAV infected ASK cells compared to the CHSE-214 cells, and was also accompanied with lower virus production in ASK. These cell responses to SAV infection may be attributed to the host (salmon) from which the virus isolate was originally isolated, assuming that SAV3 is better adapted to infect Atlantic salmon kidney cells rather than Chinook salmon embryo cells. Also, since ASK cells are macrophage-like cells they possibly produce higher amounts of IFN and Mx than CHSE-214 cells which are derived from embryonic cells.

In summary, the Q-nsP1 assay has proven to be a specific and sensitive tool for the detection of SAV RNA, and has a wide range of applications in *in vitro* studies, during experimental infection trials, for screening purposes and as a diagnostic tool. As a screening tool the Q_nsP1 assay offers a convenient means of studying potential reservoirs and vectors for SAV. The time and labour savings combined with the assay's high sensitivity and specificity enable large sets of samples to be analyzed in a short time. These are attractive features since the expected low prevalence and intensity of infection of SAV in naturally occurring populations of fish and/or potential vector species necessitates large sample sizes to be screened.

SAV; differential diagnostics

The reason why new and improved diagnostic tools are needed is because a diagnosis of SAV disease based on clinical signs and histopathology is not always straightforward. In Norway there are at least three important differential diseases which may preclude a clear-cut

diagnosis of SAV disease; IPN, CMS and HSMI. An overview of important clinical signs and histopathological features for the three diseases is given in Table 6. It appears that SAV disease, HSMI and CMS have a slightly different geographical distribution in Norway; SAV seem to be enzootic to western Norway (Rogaland, Hordaland and southern parts of Sogn og Fjordane) (Figure 8), while the majority of HSMI and CMS outbreaks have an overlapping distribution in mid Norway (Møre og Romsdal and Sør-Trøndelag). The IPNV is widespread in the farmed Atlantic salmon industry (Jarp et al., 1995), and can be readily isolated from diseased and apparently healthy fish. On the other hand, SAV is known to be notoriously difficult to isolate, especially in later stages of the disease. IPNV causes a similar pancreatic pathology as for SAV disease in salmon post-smolts but can in most cases be discriminated from PD on the basis on gross clinical signs, as well as the usual presence of catarrhal enteritis and the absence of cardiac and skeletal muscle lesions. Additionally, IPNV titres of 10^6-10^9 TCID50/g tissue and identification of IPNV using immunohistochemistry can be used to confirm an IPN disease (McLoughlin, 1997). Still, it should be noted that although heart and skeletal muscle lesions are rare features in IPN, mild cardio- and skeletal myopathy have been associated with IPN (McLoughlin, 1997).

Heart (compact and spongy layer) and skeletal muscle lesions are also the main histopathological features of fish suffering from HSMI, but this disease does not exhibit any necrosis of exocrine pancreas commonly found in SAV affected fish. Additional lesions in the liver with multifocal necrosis of hepatocytes are also associated with HSMI (Kongtorp et al., 2004a), a feature only rarely reported from SAV diseased fish (McLoughlin et al., 2002; Munro et al., 1984). As opposed to SAV and HSMI, only limited skeletal muscle lesions are described from fish with CMS (Ferguson et al., 1990). The myocardial lesions typically found in fish with CMS are not always easily differentiated from the histopathological changes

Table 6. A summary of clinical signs and histopathological lesions associated with heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS), and infections with Salmonid alphavirus (SAV) and Infectious pancreas necrosis virus (IPNV) in Atlantic salmon, including indirect or direct identification methods of SAV and IPNV. The cited references are given as numbers for convenience, and corresponds to the numbering system of publications in the reference list. The publications confirming the presence of a specific clinical sign or histopathological lesion are indicated in boldface. The publications confirming the absence of a specific clinical sign or histopathological lesion are underlined. In publications where these clinical sign or histopathological lesion are found inconsistently, the cited references are indicated in italics.

	SAV								IPN		HSMI		CMS	
	Acute 0-10 days	Ref.	Sub-acute 10-21 days	Ref.	Chronic 21-42 days	Ref.	Recovery/carrier >42 days	Ref.		Ref.		Ref.		Ref.
Histopathology														
Exocrine pancreas lesions														
Focal acinar cell necrosis	+	**82**	+/—	*82*	—	82	—	82	+	**82**	—	28, 64, 65	—	65
Diffuse acinar cell necrosis	+	**82**	+/—	*82*	—	82	—	82	+	**82**	—	28, 64, 65	—	65
Significant loss of acinar cells	—	82	+	**82**	+	**82**	+/—	*82*	+	**82**	—	28, 64, 65	—	65
Periacinar tissue fibrosis	—	82	—	82	+/—	*82*	—	82	+	**82**	—	28, 64, 65	—	65
Regeneration of acinar cells	—	82	—	82	+	**82**	+	**82**	+	**82**	—	28, 64, 65	—	65
Heart lesions									+/—	*81,* 82				
epicardium	+/—	*83, 82*	+/—	*83, 82*	+/—	*82*	+/—	*82*			+	**65, 28**	+	**29, 65**
compact myocardium	+	**83, 84, 23, 82**	+	**31, 93, 83, 84, 23, 82**	+/—	**31, 93, 83, 84, 23,** *82*	+/—	*93, 82*			+	**65, 28**	—	29, 65
spongy myocardium	+	**83, 84, 23, 82**	+	**31, 93, 83, 84, 23, 82**	+/—	**31, 93, 83, 84, 23,** *82*	+/—	*93, 82*			+	**65, 28**	+	**29, 65**
endocardium	+	**82**	+	**82**	+/—	*81,* **82**	+	**82**	+/—	*82*	+/—	*28*	+/—	*29*
Skeletal muscle lesions									+/—	*81,* 82				
red	—	23, 82	+/—	*93, 84,* 23, *82*	+/—	**93, 84,** 23, **82**	+/—	**93,** *82*			+/—	*65, 64,* **28**	(+)/—	**29,** 65
white	—	23, 82	+/—	*93, 84,* 23, *82*	+/—	**93, 84,** 23, **82**	+/—	**93,** *82*			+/—	*65,* **28**	(+)/—	**29,** 65
Liver lesions														
Multifocal hepatocytic necrosis	+/—	*83,* 82	+/—	*83, 82*	+/—	*83, 82*	+/—	*82, 83*			+	**28, 64, 65**	+/—	**29,** *65*

(Continues on next page)

Table 6 Continued

Clinical signs														
Affected fish	Typically post smolts 5-9 mo after sea transfer in May/June and September/October. However, all sizes are susceptible the whole year through†							**82**	0-3 mo after sea transfer	**126, 14**	0-9 mo after sea transfer	**136**	12-18 mo after sea transfer	**29, 13**
	0-4 kg								< 1 kg		< 1.5 kg		0,7-4 kg	
Behaviour/appearence														
swimming	Sluggish with inability to maintain normal position. Often seen congregating in corners							**82**	Hanging in cage corners, or swimming slowly	**14**	Sluggish, facing the sea current near cage wall	**65, 28**	normal	**29**
feeding	Stop feeding							**82**	+/- feed intake	**82,** 14	+/- feed intake	*65, 28*	+ feed intake	**29**
Growth	Significant reduction							**82**	+/—	*82, 14*	—	65, 28	—	29, 65
Mortalities	0-63%. Onset 2-3 weeks after drop in feeding response							**21, 43, 82**	5-20% during acute phase	**82**	0-20%	**65, 64**	0-6%	**148**
									Cumulative 0-80%	**14**				
Direct or indirect identification of the disease agent	+		+		+		+		+		—		—	
Virus isolation	+/—	**5,** *16,* **23, 20, 59,** 82, **84, 94**, *132*	+/—	**5**, *23, 59,* 82, **84, 94, 132**	+/—	*23,* 82, *132*	—	82	+	**118**				
Ab based immunostaining teqhniques	+/—	**125**, *132*	+/—	*132*	—	125, 132			+	**118**				
Serology	—	5, 23, 84, 113	+/—	*5, 23, 43, 84*	+	**5, 23, 43, 84**	+/—	**5, 43, 45**, *75, 20*	+	**118**				
RT-PCR	+	**132, unpubl. result**	+	**132, unpubl. result**	+	**132, unpubl. result**	+/—	*unpubl. result*	+	**118**				
Real-time RT-PCR	+	**47, present study**	+	**47, present study**	+	**47, present study**	+	**47, present study**	+	**136**				

† Approximately 250 days (mean) from sea transfer to outbreak (E. Brun, National Veterinary Institute, Norway, pers. comm).

found in SAV diseased fish and HSMI, although lesions seem to be restricted to the spongy myocardium in CMS (Kongtorp et al., 2004a).

Some of the clinical signs for SAV infections, CMS, IPN and HSMI are also considered as valuable indicators for a disease outbreak. For SAV, early clinical signs include a sudden decrease in feeding response, with fish that gradually become lethargic and are unable to maintain a normal horizontal position. The affected fish tend to congregate in the cage corners close to the surface in a slightly upright position, with some fish falling down to rest on the net sides or bottom. SAV infections typically affect post-smolts some 5-9 months after sea transfer in May/June and September /October, although there seems to be a change towards infections occurring throughout the year in fish of all sizes. Most of these clinical features are shared by fish affected with HSMI; appetite loss and sluggish behaviour where the affected fish typically reside close to the water surface near cage walls facing the sea current (Ferguson et al., 2005; Kongtorp et al., 2004b). Furthermore, disease outbreaks with HSMI are most common in post-smolts 5-9 months after transfer to sea. A possible difference between the clinical signs in SAV and HSMI seem to be the reduced appetite observed early in a natural outbreak of SAV, a feature not always reported from HSMI fish (Kongtorp et al., 2004b). Reduced appetite does not seem to be a prominent clinical feature in post-smolts affected with IPN, nor do they exhibit notable changes in behaviour. Furthermore, infections with IPNV typically affect post-smolts during their first three months after sea transfer (< 1 kg). Fish suffering from CMS show no obvious signs of clinical disease, especially in its acute form; only relatively large (2-4 kg) and otherwise seemingly healthy fish are affected, with no loss of appetite or abnormal swimming behaviour.

In summary, the respective characteristic clinical signs and histopathological profiles for IPN, HSMI and CMS are in most cases sufficient to differentiate between them from SAV-infections if they appear singly in fish from natural outbreaks and in its typical form. Potential

difficulties in diagnosing SAV may arise if SAV pathologies and/or IPN, HSMI and CMS occur in atypical forms or as concurrent infections. This is especially relevant considering that IPNV traditionally has been much easier to detect and isolate than the SAV, and that the pancreas lesions of the two diseases are very similar. As a consequence the prevalence and significance of SAV has probably been underestimated in the past years at the expense of IPN (similar pancreatic lesions). Implementing the Q-nsP1 real-time assay in studies of problematic case-studies could easily clarify the presence or absence of SAV in the diseased fish.

Diseases caused by SAV; - are they different?

Today, we know that there are at least three subtypes of the Salmonid alphavirus causing mortalities in salmonid farming (**Paper I**), but are they manifested as three different diseases? In a historical perspective, pancreas disease has been used to denote disease conditions caused by severe infections of SAV in salmon farming in the British Isles and Norway, whereas sleeping disease is caused by SAV infection in rainbow trout farming in France. However, the recent discovery of SAV3 somewhat complicates this picture because we now have three different subtypes of SAV, but there are only two names for the diseases caused by these subtypes. Although PD always has been, and still is, treated as a uniform disease across Europe (the British Isles and Norway), it is important to emphasise that the SAV3 is in fact equally distant from SAV1 as SAV2 (**Paper I**), and should be acknowledged as a separate disease agent different from SAV1 (and SAV2). In terms of disease-causing abilities, previous descriptions of SAV1 and SAV3 are however not sufficiently different to separate them as distinctive diseases, but more studies of pathogenesis will be needed to clarify this issue. Such comparative studies of the biological properties of SAV should not only include SAV1 and SAV3, but also SAV2 because of the striking histopathological similarities of SD

(SAV2) and PD (SAV1, SAV3). Despite the fact that PD and SD share identical histopathological lesions, they have always been mentioned and treated as two distinct and separate diseases. Principal hosts are rainbow trout for SD (*O. mykiss* > *S. salar* > *S. trutta*) and Atlantic salmon for PD (*S. salar* > *O. mykiss*), but cross-infections can occur (Boucher et al., 1995; Olsen and Wangel, 1997; Villoing et al., 2000b). SD was initially described from rainbow trout and given its name based on the peculiar behaviour of affected fish. The origin of the name PD, on the other hand was based on the severe exocrine pancreas lesions observed in diseased Atlantic salmon in the original description by Munro et al. (1984). The two disease conditions were thus named based on two very different criteria; a) clinical behaviour ->SD, and b) histopathological lesion ->PD. However, the histopathology of the two diseases is strikingly similar, with identical lesions in the key organs; exocrine pancreas, heart muscle and skeletal muscle. These are all lesions that would classify the two diseases together rather than separating them. This is also supported by Boucher et al. (1996) who concluded that the difference between PD and SD lesions in experimentally infected fish were more quantitative rather than qualitative supports this view.

At first glance the clinical signs observed in fish with SD or PD seem quite different; SD is characterized by the behaviour of affected fish who tend to "rest" at the bottom of tanks, while lethargic PD fish are seen aggregating in the water surface facing the cage wall or corners. Although these are the typical clinical observations referred to for SD and PD, they are not contradictory. The characteristic "sleeping" condition in SD affected fish can only be observed in tanks or basins where the bottom surface is visible. When handled they will swim for a very short time, simply as an escape response, before returning to the bottom, again lying on their sides. In deeper or muddier farm basins any fish lying on their sides is not readily seen, and this behaviour is thus overlooked. In these cases only the lethargic fish staying near the surface waters are observed. This latter example is very similar to the typical

behaviour observed in clinically diseased post-smolts in net-pens during a natural PD outbreak. Here, lethargic PD fish tend to stay close to the water surface near cage walls, and some fish are seen resting or hanging on the side of the net-pens. Furthermore, McLoughlin et al. (2002) pointed out that PD fish can appear dead on the bottom of the cage, but swim away when handled, an observation identical to the "sleeping" behaviour seen in SD affected rainbow trout. Apparently lethargic fish lying on their sides on the net floor is also observed from fish suffering from HSMI (Ferguson et al., 2005), indicating that this behaviour is not exclusive or otherwise characteristic for SAV2 infections. Thus, the differences in the disease appearance of PD and SD, if there are any, would then simply reflect the different farming conditions for the affected hosts from which SD and PD are commonly reported from. Hypothetically, if extensive farming of salmon and rainbow trout were to overlap in time and place a situation where either PD or SD or both co-occurred, a confirmatory SD or PD-diagnose based on clinical signs would be impossible to make.

In summary, the behaviour, clinical signs and histopathological lesions in salmonids suffering from PD (SAV1, SAV3) and SD (SAV2) are remarkably similar and as disease conditions they should be considered identical. Therefore, an umbrella term for the historical names PD and SD, which merely refers to the virus species involved rather than the clinical signs ("sleeping disease") and histopathological lesions ("pancreas disease") is proposed; Salmonid Alphavirus Disease (SAVD). The term SAVD in salmonids would then include the same histopathological lesions in the key organs exocrine pancreas, heart- and skeletal muscle as previously described from the former SD and PD, but the clinical signs would be more detailed and include the full range of behavioural characteristics from both PD and SD. Because clinical signs and histopathology alone cannot unequivocally discriminate between subtypes of SAV (see above) a more correct term would be to use SAVD, and when needed (and if possible), a more specific assignment of the disease agent (SAVD subtype-1, -2 or -3)

may be given. At present, the only way to further assign SAVD to one (or more) of the three subtypes of SAV, is to perform specific tests such as real-time RT-PCR and/or sequencing studies. However, introducing a new term common for PD and SD present some drawbacks; PD and SD are now well-established and commonly accepted labels or names for these disease conditions, and it can be argued that by introducing a new term (SAVD) only contribute to the confusion around SAV subtypes and the diseases they are causing. Nevertheless, it may be appropriate to adopt the name SAVD (with accompanying subtype), at least in a scientific content, to increase the accuracy in denoting SAV-induced diseases.

Conclusions

- **Paper I** comprises the first presentation of the nucleotide sequence from a new subtype of Salmonid Alphavirus (SAV). This new subtype, SAV3, is only found in Norwegian aquaculture of *Salmo salar* and *Oncorhynchus mykiss*.
- Sequence analyses of 20 SAV3 isolates from Norway have shown that SAV3 is a genetic homogenous population, and seem to have an enzootic focus on the west coast of Norway (**Paper II**). The recent disease outbreaks in northern parts of Norway are best explained by transport of SAV3 infected fish from smolt producers located in the enzootic region.
- TaqMan based real-time RT-PCR protocols were developed for detecting RNA from all subtypes of SAV, and can be used to differentiate and quantitate any subtype of salmonid alphavirus within the host (**Paper III**). Using real-time RT-PCR for detection of SAV not only saves time and labour, but also offers increased sensitivity and specificity compared to traditional diagnostic methods.

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Paper I

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New subtype of salmonid alphavirus (SAV), *Togaviridae*, from Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* in Norway

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ABSTRACT: In Europe, 2 closely related alphaviruses (*Togaviridae*) are regarded as the causative agents of sleeping disease (SD) and salmon pancreas disease (SPD): SD virus (SDV) has been isolated from rainbow trout *Oncorhynchus mykiss* in France and the UK, while SPD virus (SPDV) has been isolated from salmon *Salmo salar* in Ireland and the UK. Farmed salmonids in western Norway also suffer from a disease called pancreas disease (PD), and this disease is also believed to be caused by an alphavirus. However, this virus has not yet been characterised at the molecular level. We have cultured a Norwegian salmonid alphavirus from moribund fishes diagnosed with cardiac myopathy syndrome (CMS) and fishes diagnosed with PD. The virus has also been found in salmon suffering from haemorrhagic smolt syndrome in the fresh water phase. The genomic organisation of the Norwegian salmonid alphavirus is identical to that in SPDV and SDV, and the nucleotide sequence similarity to the other 2 alphaviruses is 91.6 and 92.9%, respectively. Based on the pathological changes, host species and the nucleotide sequence, we suggest naming this virus Norwegian salmonid alphavirus (NSAV). Together with SPDV and SDV it constitutes a third subtype of salmonid alphavirus (SAV) species within the genus Alphavirus, family *Togaviridae*.

KEY WORDS: Norwegian salmonid alphavirus · Virus characterisation · Pancreas disease

INTRODUCTION

In Europe, 2 closely related alphaviruses (*Togaviridae*) have been described from farmed salmonids. The salmon pancreas disease virus is the causative agent of pancreas disease in farmed Atlantic salmon *Salmo salar* in Ireland and Scotland (Nelson et al. 1995, McLoughlin et al. 1996, Rowley et al. 1998, Welsh et al. 2000, Weston et al. 1999) and sleeping disease virus the aetiological agent of sleeping disease in France and the UK (Boucher et al. 1995, Castric et al. 1997, Branson 2002, Villoing et al. 2000, Graham et al. 2003). In North America, pancreas disease was first described in 1987 (Kent & Elston 1987). In Canada in 1996, a togavirus-like agent was observed together with the infectious salmon anemia virus in association with a disease called haemorrhagic kidney syndrome (HKS) (Kibenge et al. 2000). However, this togavirus-like agent has not been identified and characterised.

Farmed salmonids (*Salmo salar* and *Oncorhynchus mykiss*) in Norway also suffer from a disease called pancreas disease, that seems to be caused by an alphavirus (Christie et al. 1998 and unpubl. data, Weston et al. 2002, 2003), but this virus has not yet been properly characterised at the molecular level. Affected fish often show an impaired swimming performance and loss of appetite. They tend to congregate at the surface in the cage corners, unable to maintain a normal position. Disease outbreaks have only been observed in sea water and usually 5 to 7 mo after the smolts have been transferred to the sea. Histopathological findings may include total or severe degeneration of exocrine pancreas and always various degrees of myopathy of skeletal and heart muscles (A. Nylund

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pers. obs., Poppe & Rimstad 1989). *Alphavirus* spp. have also been found, using RT-PCR and sequencing, in moribund farmed salmon in Norway diagnosed with cardiac myopathy syndrome, CMS (A. Nylund pers. obs.) and in salmon suffering from haemorrhagic smolt syndrome (HSS) in the fresh water phase (Nylund et al. 2003).

The majority of cases in which *Alphavirus* spp. have been detected associated with disease in salmon and rainbow trout have been in Hordaland and Sogn og fjordane (western Norway), but recently a few cases have been seen in northern Norway (Nordland, Troms and Finnmark) (A. Nylund pers. obs.). The genomes of the viruses from northern Norway have been partially sequenced and are identical to those from western Norway. This means that *Alphavirus* spp. have been found in the majority of Norwegian counties with salmon farming.

The complete genomes of SPDV from Ireland (Isolate F93-125) and SDV from France (Isolate S49P) have been sequenced and consist of about 12 kb (Weston et al. 2002). No other isolates have been completely sequenced, but partial sequencing of selected nonstructural and structural protein genes indicate that SPDV and SDV from the UK are identical to those sequenced from Ireland and France, respectively (Graham et al. 2003, Weston et al. 2003). Included in the studies by Weston et al. (2002, 2003) are also 2 Norwegian alphavirus isolates, PD97-N2 and PD97-N3 (supplied by K. E. Christie, Intervet, Bergen), originating from diseased salmon and rainbow trout, respectively. Both isolates were partially sequenced by Weston et al. (2003) from a virus harvested from Chinook salmon embryo CHSE-214 cell cultures, and the sequences were found to belong to the SPDV Alphavirus type (Subtype 1). It will, however, be shown in this study that the PD97-N2 and PD97-N3 isolates are in fact a new, different and distinct Norwegian subtype of salmonid alphavirus.

The first complete genome of the Norwegian salmonid alphavirus subtype (NSAV) from 4 separate outbreaks of disease, 2 diagnosed with cardiac myopathy syndrome and 2 with pancreas disease, are presented herein. The sequences are compared with those of the SDV and the SPDV from France and Ireland. The phylogenetic relationship between Norwegian salmonid alphavirus, SPDV and SDV is discussed. It is suggested that the Norwegian isolates be given status as a separate subtype, Norwegian salmonid alphavirus (NSAV), within the salmonid species alphavirus (Weston et al. 2002).

MATERIALS AND METHODS

The Norwegian salmonid alphavirus (NSAV) was collected from salmon and rainbow trout at 4 different farms in western Norway (Table 1). The first isolate was collected in 1997 from rainbow trout with pancreas disease (PD) in Hordaland (Isolate PD97-N3, Weston et al. 2002, 2003, K. E. Christie unpubl. data) and cultured in CHSE-214 cells. We collected 2 isolates (SavH10/02 and SavH20/03) from Atlantic salmon *Salmo salar* diagnosed with cardiac myopathy syndrome (CMS) in 2002 and 2003 in Hordaland. The 4th isolate (SavSF21/03) was collected from salmon diagnosed with PD in 2003 in Sogn og Fjordane. Selected tissues (gills, heart, pancreas, kidney and somatic muscle) were frozen in liquid nitrogen for storage at –80°C.

Tissue homogenates of the original material from which the 2 isolates PD97-N2 and PD97-N3 were isolated, and later cell culture passages of the same 2 isolates (supplied by K. E. Christie), have also been included in the present study. The PD97-N2 isolate was collected from Atlantic salmon in Hordaland in 1997 (Christie et al. 1998). The tissue homogenates and the cell culture passages had been stored in liquid nitrogen. To establish if the Irish/UK SPDV subgroup was present in Norway or if there could have been laboratory contamination of the cell cultures with an isolate (F93-125) from Ireland, we screened Passages 5, 6 and 12 of Isolate PD97-N2 and Passages 4, 6 and 12 of Isolate PD97-N3 for the presence of salmonid alphavirus (SAV), and sequenced a 899 bp segment from the structural genes from all passages and the tissue homogenates.

Cell culture. The Norwegian salmonid alphavirus was first isolated, using CHSE-214 cells, from tissue homogenates of heart and kidney from salmon suffering from CMS (SavH10/02). The CHSE-214 cells were cultured in 15 cm^2 tissue culture flasks (Nunc) at 20°C in Eagles minimum essential medium (EMEM) (Sigma) supplemented with 10% foetal bovine serum (FBS) (10% v/v), L-glutamine (4 mM) and gentamicin (50 µg ml^{-1}). The cells were then subcultured for 7 to 10 d

Table 1. Salmonid alphaviruses (NSAV) included in this study. PD97-N3 and the 3 Sav isolates were collected from 4 different farms in western Norway. *S*: *Salmo salar*; *O*: *Oncorhynchus mykiss*

Virus	Year	Country	Locality	Host	Accession No.
PD97-N2	1997	Norway	Osterøy	*S*	
PD97-N3	1997	Norway	Osterøy	*O*	AY604237
SavH10/02	2002	Norway	Øygarden	*S*	AY604236
SavH20/03	2003	Norway	Sotra	*S*	AY604235
SavSF21/03	2003	Norway	Gulen	*S*	AY604238
F93-125	<1995	Ireland	West Ireland	*S*	AJ316244
S49P	?	France	Atlantic coast	*O*	AJ316246

until the tissue flasks were covered with a 60 to 80% confluent monolayer. Homogenates from SMAV-infected tissues were diluted 1:100 in phosphate buffered saline and incubated for 1 h at 15°C in cell culture flasks with the monolayer of CHSE-214 cells. The inoculum was then removed and replaced by supplemented EMEM as described above, but with 2% FBS. The cells were incubated for 4 to 8 wk or until a cytopathic effect (CPE) could be observed. The cultures were supplied with fresh media at intervals of 1 to 2 wk.

RNA extraction. RNA was extracted from infected cell cultures and tissues (heart and kidney) and reverse transcribed into cDNA as described by Devold et al. (2000).

RT-PCR. PCR products of the 4 Norwegian alphavirus isolates were obtained using primers directed against conserved areas of the SPDV (F93-125, Accession No. AJ316244) and SDV (S49P, Accession No. AJ316246) genome. The same primers were also used for sequencing.

The primers F4 (5'-AGC GAC TCC CAG ACG TTT ACG-3') and R1 (CGG TTT ATC ACT GCT TCG TAC GA-3') amplify an 899 bp fragment in the E2-6K-E1 junction. This primer set was used to obtain a fragment from tissue homogenates and from different cell culture passages of the 2 SAV isolates PD97-N2 and PD97-N3. The PCR amplifications were performed in a total volume of 50 µl using 2 µl of template cDNA, and the reaction mixture consisted of: 1× PCR buffer, 0.8 mM dNTP, 0.2 µM of the reverse and forward primer, 2 U of thermostable DNA polymerase (Qiagen), and dH_2O. The PCR conditions were as follows: after an initial 5 min denaturation step at 95°C, samples were taken through 35 amplification cycles, each consisting of a 30 s denaturation step at 94°C, a 30 s primer annealing step at 55°C, and a 1 min and 30 s extension step at 72°C. A prolonged extension step of 10 min at 72°C completed each reaction.

Real-time RT-PCR. Real-time RT-PCR assays were designed to separate the Norwegian salmonid alphaviruses and the SPDV isolate (F93-125). These assays determined whether the SPDV isolate was present in addition to the NSAV. They have also been used to screen for the presence of NSAV in fish diagnosed with CMS. The design of the primers and TaqMan TM probes was carried out using Primer Express Software (PE Applied Biosystems). The primer pair and probes specific for SPDV Subtype 1 were 5'-ACAGTGAAATTCGACAAGAAATGC-3' (forward), 5'-TGGGAGTCGCTGGTGAAAGT-3' (reverse) and FAM-5'-AGAGCGCTGACTCGGCAACCGT-3' –MGB (probe), whereas the primer pair and probe specific for NSAV were 5'-CAGTGAAATTCGATAAGAAGTGCAA-3' (forward), 5'-TGGGAGTCGCTGGTAAAGGT-3' (reverse) and FAM-5'-AGCGCTGCCCAAGCGACCG-3' –MGB (probe). These 2 assays amplify a region in the E2 gene. All primers and probes were obtained from PE Applied Biosystems. The TaqMan assays were performed with 2 µl of cDNA, 900 µM of each primer, and 200 µM of probe in a total volume of 50 µl using the TaqMan Universal PCR Master Mix w/AmpErase® UNG (PE Applied Biosystems). Amplification and fluorescence detection were performed with the ABI Prism 7000 sequence detection system instrument as recommended by the manufacturer (PE Applied Biosystems).

Sequencing. The PCR products were purified using Qia-quick PCR purification columns (Qiagen) and then sequenced using the Big Dye terminator sequencing kit (Applied Biosystems). The complete genomes from all 4 salmonid alphaviruses were sequenced in both directions (SavH20/03–AY604235, SavH10/02–AY604236, PD97-N3–AY604237, SavSF21/03–AY604238). Sequence data were assembled with the help of Vector NTI software (InforMax) and GenBank searches were performed using BLAST (2.0).

The sequences of the 4 NSAV isolates were aligned in Clustal X, in Vector NTI software, with full length sequences from SPDV (F93-125) and SDV (S49P) already available in the EMBL nucleotide database. To perform pairwise comparisons between the different SAV isolates, the multiple sequence alignment editor GeneDoc (available at www.psc.edu/biomed/genedoc) was used. In addition to software analysis of the sequences, polymorphic regions were manually aligned and compared.

Phylogeny. The evolutionary relationships between alphaviruses have been presented by Powers et al. (2001) using partial E1 envelope glycoprotein gene sequences and complete structural polyprotein sequences. We performed a study of the nonstructural polyprotein amino acid sequences of a selection of alphaviruses including the NSAV isolates, and obtained a similar phylogeny (data not presented). In the present study, a phylogenetic analysis of a selection of SAV isolates using the complete nucleotide genome sequence was performed: Accession Nos. AJ316244 (F93-125), AJ316246 (S49P), AY604235 (SavH20/03), AY604236 (SavH10/02), AY604237 (PD97-N3), AY604238 (SavSF21/03). The alignment was manually adjusted using the sequence alignment editor GeneDoc (available at www.psc.edu/biomed/genedoc) and gaps were deleted before analysing. The analyses were performed using TREE-PUZZLE 5.0 (available at www.tree-puzzle.de). TREE-PUZZLE reconstructs phylogenetic trees from molecular data by maximum likelihood, and computes maximum likelihood distances and branch lengths. In this study, 1000 quartet puzzling (QP) steps were carried out. The QP tree search

estimates support values for each internal branch. Branches showing QP reliability from 90 to 100% can be considered very strongly supported. Branches with lower reliability (>70%) can, in principle, be trusted. Phylogenetic trees were drawn using TreeView (Page 1996).

RESULTS

The almost complete genome of 4 alphaviruses (Norwegian salmonid alphavirus) associated with disease in Norwegian farmed Atlantic salmon *Salmo salar* and Rainbow trout *Oncorhynchus mykiss* in sea water have been sequenced. The 4 NSAV sequences deposited in the GenBank lack 8 to 53 nucleotides (nt) at the 5'-end of the first open reading frame (ORF). Two isolates, SavH10/02 (Accession No. AY604236) and SavH20/03 (Accession No. AY604235), were obtained from salmon diagnosed with cardiac myopathy syndrome (CMS) by the Norwegian Veterinary Service; 1 (SavSF21/03, Accession No. AY604238) was associated with 'classical' pancreas disease (PD), and 1 was isolated from rainbow trout (PD97-N3, Accession No. AY604237). Of these isolates, 2 (PD97-N3 and SavH10/02) were sequenced after growing in cell culture, while the latter 2 (SavH20/03 and SavSF21/03) were sequenced using RNA extracted from host tissues (mainly heart). SavH10/02 and N3-1997 grew well in cell cultures (CHSE-214), but produced no prominent cytopathic effect (CPE).

The genome organisation of the 4 NSAV isolates is similar to that of the Irish salmon pancreas disease virus, SPDV (Isolate F93-125), and the French sleeping disease virus, SDV (Isolate S49P). However, comparisons over the complete genome between the Norwegian isolates (NSAV), SPDV (F93-125) and SDV (S49P) revealed large sequence differences (Tables 2 & 3). Comparing 11 742 nucleotides from NSAV with SPDV and SDV revealed 91.6 and 92.9% similarity, respectively.

The first ORF encoding the nonstructural proteins (nsP1, nsP2, nsP3 and nsP4), in the NSAV isolates is shorter than that of SPDV and SDV. The ORF of the 4 NSAV isolates has the same length (7761 nt). The major reason for the size difference is insert/deletions in nsP3 (Table 4). The sequence similarity over the nonstructural subgenomic part is higher than 99% between the NSAV isolates, while their similarity to SPDV and SDV is 90 and 92%, respectively. The nonstructural polyproteins of the 4 NSAV isolates are predicted to be 2587 amino acids. The amino acid (AA)

Table 2. Salmonid alphavirus (SAV). Percent nucleotide (nt) sequence similarities between the 3 subtypes in Europe, comparing the different ORFs (open reading frame of SavH10/02 isolate) on the genomic strand. SPDV: salmon pancreas disease virus; SDV: sleeping disease virus

Subtype	Isolate	NSAV SavH10/02								
		nsP1[a]	nsP2	nsP3	nsP4	C	E3	E2	6K	E1
NSAV	N3-1997	99	99	99	100	99	99	99	99	100
NSAV	SavH20/03	100	99	99	99	100	100	99	100	100
NSAV	SavSF21/03	99	100	99	100	99	99	99	100	100
SPDV	F93-125	94	91	85	92	91	89	89	94	93
SDV	S49P	95	93	88	94	88	92	92	94	93
ORF/nt		1631[a]	2577	1674	1829	845	210	1316	206	1385

[a]A few nucleotides are missing at the beginning of the ORF

Table 3. Salmonid alphavirus (SAV). Percent amino acid (aa) sequence similarities between the 3 subtypes in Europe, comparing the different proteins

Subtype	Isolate	NSAV SavH10/02								
		nsP1[a]	nsP2	nsP3	nsP4	C	E3	E2	6K	E1
NSAV	N3-1997	100	99	99	100	100	98	99	98	100
NSAV	SavH20/03	100	100	100	100	100	100	100	100	100
NSAV	SavSF21/03	100	100	99	100	100	98	99	100	100
SPDV	F93-125	95	96	88	97	95	94	95	97	98
SDV	S49P	97	97	90	98	88	95	94	95	96
No. of aa		543	859	558	609	281	71	438	68	461

[a]A few amino acids are missing at the beginning of the protein

Table 4. Salmonid alphaviruses (SAV). Location of gaps/insertions in complete genome in relation to complete genome of S49P (SDV), Accession No. AJ316246, which is used as reference

Subtype	Isolate	Insert	Insert	Gap	Gap	Gap	Gap	Gap	Gap
NSAV	SavH10/02	–	24 nt/nsP3	3 nt/nsP3	12 nt/nsP3	27 nt/nsP3	3 nt/C	3 nt/C	3 nt/E1
SPDV	F93-125	3 nt/nsP1	24 nt/nsP3	3 nt/nsP3	–	–	–	3 nt/C	3 nt/E1
SDV	Position	1662-1663	5373-5374	5595-5599	5695-5707	5715-5743	8177-8181	8486-8490	11723-11727

sequence similarity is higher than 99% between the NSAV isolates, while their similarity to SPDV and SDV is 95% and 96%, respectively.

The cleavage sites in the NSAV nonstructural polyproteins were deduced from amino acid sequence homology with the SPDV and the SDV. The size of nsP1, nsP2, nsP3 and nsP4 are 561, 859, 558 and 609 AA, respectively. The sequence similarities between the nonstructural proteins from the different salmonid alphaviruses are given in Tables 2 and 3. The nucleotide sequence similarity between the different nonstructural proteins of the 3 virus groups varies from 85 to 95%, with the largest difference between NSAV and SPDV in nsP3 and the highest similarity between NSAV and SDV in nsP1 (Table 2). The ORF of Proteins nsP2 and nsP4 has the same length in all 3 (NSAV, SPDV and SDV) of the salmonid alphaviruses. As in other alphaviruses the nsP4 protein contains the conserved motif GDD at Residues 466 to 468 present in all 3 salmonid alphavirus subtypes (SDV, SPDV and NSAV). The SPDV has longer ORFs than the other 2 SAV subtypes when comparing nsP1 and nsP3 (Table 4). The opal termination codon found in some alphaviruses between the nsP3 and nsP4 proteins (Strauss & Strauss 1994) is not found in the NSAV isolates, where the nsP3 ends with the codon GGG (gly). The TGA codon is replaced by a sense codon CAA (glutamine) followed by CGA (arginine), and hence 2 nucleotides are changed from the tetranucleotide TGAC.

The second ORF of the 4 NSAV isolates, encoding the structural proteins, is 3960 nt long including the stop codon (TAA). Hence, it is slightly shorter than the ORF of both the SPDV and SDV. The NSAV isolates show more than 99% nucleotide identity over their structural polyprotein region, while their similarity to SPDV and SDV is 91 and 92%, respectively. When the individual structural protein genes of the NSAV isolates are compared with SPDV and SDV, the nucleotide similarities vary from 88% for the capsid protein gene (SDV) to 94% for the 6K gene (SPDV and SDV) (Table 2). The nucleotide variation is fairly evenly distributed. However, 2 gene regions with relatively high nucleotide difference occur in the capsid gene and 1 region at the C terminus of the E1 protein. The similarity at the amino acid level between the NSAV isolates and SDV and SPDV vary from 88% for the capsid protein (SDV) to 98% for the E1 protein (SPDV) (Table 3). The structural polyproteins of the NSAV isolates are comprised of 1320 amino acids and show highest similarity to SPDV, with the exception of the E3 gene, which is most similar to E3 from the SDV isolate (Table 3). The ORF of Proteins E3, E2, and 6K is the same length in all 3 (SMAV, SPDV and SDV) of the salmonid alphaviruses. The ORFs of C and E1 from SDV are longer than that of NSAV and SPDV.

The first ORF of the NSAV isolates is followed by a non-translated region of 38 nt before the start of the second ORF encoding the structural proteins. This non-translated region has a nucleotide sequence identical to that of the SDV (S49P). One of the conserved nucleotide sequence elements (CSE) of alphaviruses, CSE3 (24 nt), is also present in this region of the NSAV isolates, and includes the last 12 nt from the end of the nsP4 sequence and the first 12 nt of the non-translated region. The CSE3 sequence of the NSAV isolates is identical to that found in S49P (Villoing et al. 2000) and differs by 1 nt from that of the SPDV isolate F93-125 (Weston et al. 2002).

The CSE2 of the NSAV isolates were identified by aligning nsP1 with the complete sequence of Sindbis virus (Accession No. NC_001547). The nucleotide and amino acid sequence similarities of nsP1 from the NSAV isolates compared to the SIN nsP1 are 49 and 41%, respectively. However, the nucleotide and amino acid sequence similarities of the CSE2 motif of the NSAV and SIN are 74 and 71%, respectively. CSE2 is a 48 nt sequence stretching from Positions 153 to 200 on the complete genome of SDV (Accession No. NC_003433) (Table 5). In the NSAV isolates, 2 hypothetical stem-loop structures are suggested for the CSE2 (Fig. 1), and were constructed using Mfold 3.1 (Zuker 2003).

The F4-R1 amplicons, from the tissue homogenate of Atlantic salmon with NSAV PD97-N2, were almost identical to the same gene from the other 4 NSAV isolates. However, in Cell Culture Passage 5, the Norwegian SAV, PD97-N2, was found to have been substituted by the Irish SPDV (F93-125). Use of the specific real time RT-PCR assay for Isolate F93-125 could not detect this isolate before Passage 5.

Table 5. Alphavirus. The '51-nucleotide' conserved sequence element (CSE2), showing linear nucleotide sequences of 4 isolates sequenced through this region. Upper row is CSE2 sequence from Sindbis virus, SIN (Niesters & Strauss 1990), the other 3 viruses are salmonid alphaviruses (NSAV, SDV and SPDV). Nucleotides that are similar to Sindbis virus CSE2 sequence are represented by dots. aa = amino acids. There are 2 deletions (–) in the salmonid alphaviruses compared to SIN. Nucleotide numbers refer to full length sequences of Sindbis virus (NC_001547) and SDV (AJ316246)

aa		Q	V	T	P	N	D	H	A	N	A	R	A	F	S	H	L	A	
nt (SIN)	155																	205	
SIN	CAG	CAG	GUC	ACU	CCA	AAU	GAC	CAU	GCU	AAU	GCC	AGA	GCA	UUU	UCG	CAU	CUG	GC	CAG
NSAV	..A	...	...	G-.	...	.C-	...	...	..C	GCC	...	...	..U	..C	..C	..C	U..	..	...
SDV	..A	.C.	...	G-.	...	.C-	...	...	..C	GCC	...	...	..U	..C	..C	..C	U..	..	...
SPDV	..A	U..	...	G-.	.U.	.C-	...	...	..C	GCC	...	...	..U	..C	..C	..C	U..	..	...
nt(SDV)		153																200	
aa	N	R	S		S	N	D	H	A	A	A	R	A	F	S	H	L	A	

Complete genome sequences of the 4 NSAV, the SPDV and SDV were used to compare the genetic relationships between these viruses (Fig. 2). The genetic distance tree shows that the salmonid alphaviruses constitute 3 different subtypes within this species.

DISCUSSION

This paper comprises the first presentation of the complete nucleotide sequence from a new subtype of salmonid alphavirus. This new subtype has, so far, only been found in Norwegian aquaculture of salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*. Including this Norwegian salmonid alphavirus, the SAV group now consists of 3 distinctly different but related viruses: (1) the sleeping disease virus (SDV) isolated from rainbow trout in France and UK (Villoing et al. 2000, Graham et al. 2003); (2) the salmon pancreas disease virus (SPDV) isolated from salmon in Ireland and the UK (Weston et al. 1999, Welsh et al. 2000); (3) the Norwegian salmonid alphavirus (NSAV) isolated from salmon and rainbow trout in Norway (Christie et al. 1998, and present study). NSAV isolates cause heart and skeletal muscle myopathy and have been associated with diseases like pancreas disease (Christie et al. 1998), haemorrhagic smolt syndrome (Nylund et al. 2003), and cardiac myopathy syndrome (present study). The detection of NSAV from smolt with HSS was probably an accidental finding and not the causative agent of this disease (cf. Nylund et al. 2003).

Experimental challenges of salmon using NSAV isolates seem to result in pathological changes associated with PD (Poppe & Rimstad 1989, Christie et al. 1998, authors' pers. obs.) and CMS in western Norway (Ferguson et al. 1990, Nylund 2001). Because of a lack of sensitive and specific diagnostic tools, these 2 diseases have been confused in salmon aquaculture in western Norway. However, NSAV isolates are not commonly found in salmon aquaculture north of western Norway, except for a few documented cases in which the virus

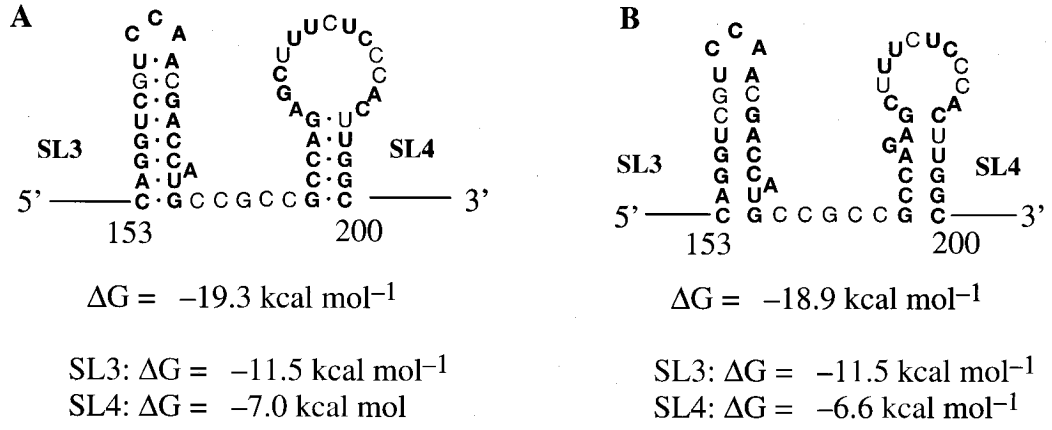

Fig. 1. Norwegian salmonid alphavirus (NSAV), showing 2 possible hypothetical stem-loop structures of conserved sequence element CSE2. ΔG values calculated at 15°C. Nucleotides that seem to be conserved between NSAV isolates and Sindbis virus are in boldface; numbers refer to complete genome of sleeping disease virus (Accession No. AJ316246). Stem-loop structures (SL3 and SL4) after Frolov et al. (2001)

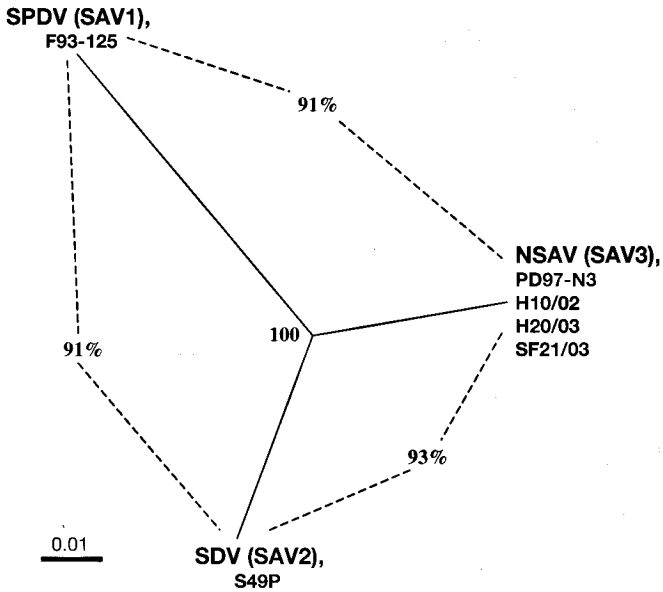

Fig. 2. Salmonid alphaviruses (SAV). Genetic distance of the SAV subtypes in relation to each other. Evolutionary relationship based on alignment of complete genome (11720 nucleotides) of 6 SAV isolates including all 3 subtypes (SAV1, SAV2 and SAV3). Scale bar: number of nucleotide substitutions as a proportion of branch length. Percent nucleotide similarity between the subtypes is shown. SPDV: salmon pancreas disease virus; SDV: sleeping disease virus

was most likely introduced to northern Norway via transport of infected smolts (A. Nylund pers. obs.). There have also been cases of CMS in western Norway, where we have been unable to detect NSAV using the 2 real-time PCR assays. These assays are very sensitive and can detect a carrier status in infected salmon. Hence, the reports of NSAV in salmon suffering from CMS in western Norway could be accidental findings, or merely reflect the lack of satisfactory official diagnostic methods for separating pancreas disease from CMS in Norway. Except for a few cases in which NSAV was present in fishes diagnosed with CMS, CMS may be considered as a separate disease not caused by the NSAV.

Analysis of the genomic sequences from the 4 NSAV isolates shows that the NSAV possesses a genome organisation identical to that observed for the other 2 SAV isolates, SPDV and SDV (Weston et al. 2002), and for mammalian alphaviruses (Strauss & Strauss 1994). NSAV, SPDV and SDV are also very similar at both the nucleotide level and the amino acid sequence level. The nucleotide sequence identity of the 3 viruses is above 90% over the complete genome and the amino acid identity about 95%. The NSAV isolates are more similar to the SDV (93%) than the SPDV (91%). The similarity to the mammalian alphaviruses is much lower, and a phylogenetic analysis based on the amino acid sequence of the structural polyproteins show that the SAV isolates constitute a distinct species (salmonid alphavirus, SAV) within the genus Alphavirus of the family *Togaviridae* (Powers et al. 2001, cf. Weston et al. 2002).

Most alphaviruses contain an opal codon (UGA) in the ORF between nsP3 and nsP4, and this is leaky when it is followed by a single C residue downstream, i.e. UGAC (Strauss & Strauss 1994). This opal codon is believed to regulate the production of nsP4 in infected cells, but it is not present in the SAV isolates. The conserved GDD motif characteristic of viral RNA polymerases (Strauss & Strauss 1994) is, on the other hand, present in the 3 subtypes of SAV.

Although the SAV isolates show no close relationship to any of the mammalian alphaviruses, they probably contain all the conserved sequence elements believed to play important roles in virus replication. Comparisons of Alphavirus genomes and mutagenesis of conserved RNA elements have identified a number of *cis*-acting sequences including (1) a 19 nt conserved sequence element (CSE4) which immediately precedes the 3'-terminal poly(A) tract, (2) a 24 nt sequence (CSE3) that spans the start site of the subgenomic RNA, (3) the 5'UTR with low overall homology between different alphaviruses but a conserved predicted secondary structure, and (4) the 51 nt (CSE2) believed to enhance RNA replication (Niesters & Strauss 1990, Strauss & Strauss 1994, Frolov et al. 2001). Since the sequences of the NSAV isolates are not complete at the 5'- and 3'-ends, we have only been able to locate what we believe are the CSE2 and CSE3. These CSE show a high sequence similarity to the mammalian alphaviruses.

It has been suggested, based on nucleotide sequences and reactivities of SPDV- and SDV-derived monoclonal antibodies with SPDV and SDV isolates, that NSAV, PD97-N2 and PD97-N3 are almost identical to the Irish SPDV isolate, F93-15 (Weston et al. 2002, 2003). As the present study shows, the tissue homogenates from which these 2 isolates (PD97-N2 and PD97-N3) were obtained contained only SAV isolates that are almost identical to the other Norwegian NSAV isolates. However, the PD97-N2 isolate is identical to SPDV (F93-125) in Passages 5 and 6 (material supplied by K. E. Christie). We believe this change could be a result of contamination by the Irish isolate (F93-125) that was in the laboratory at the time of isolation. Another possible explanation is that both isolates were present at the same time in the salmon and that 1 isolate became dominant after Passage 4. However, we have not been able to detect the F93-125 isolate in the tissue homogenates using a sensitive and specific real time RT-PCR assay. Nor has the F93-125 isolate or isolates similar to this been found in farmed salmonids from Norway. All cell culture passages (supplied by

K. E. Christie) of Isolate PD97-N3 were of the NSAV type. Since this isolate also emerged as nearly identical to the Irish SPDV (F93-125) in the studies of Weston et al. (2002, 2003), the change of isolate must have occurred at a later point.

The present study supports the classification of SPDV, SDV and NSAV as a new species, salmonid alphavirus (SAV), in the genus *Alphavirus* of the family *Togaviridae*, as suggested by Weston et al. (2002). This new species can further be divided into 3 distinct subtypes: (1) SPDV or SAV1 from salmon in Ireland and the UK (Weston et al. 2002); (2) the SDV or SAV2 from rainbow trout in France and the UK (Weston et al. 2002), and the Norwegian NSAV or SAV3 from salmon and rainbow trout in Norway (present study).

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

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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 (± 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

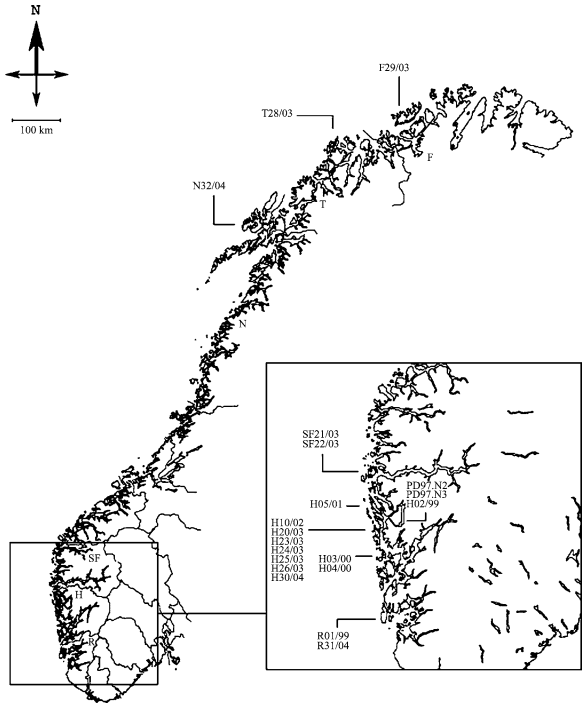

Fig. 1. The coastline of Norway. Locations where the NSAV isolates were collected are indicated. The isolate prefix (SAV-) is left out for convenience. Counties that are discussed in the text are indicated on the map as follows: *F* = Finnmark, *T* = Troms, *N* = Nordland, *SF* = Sogn og Fjordane, *H*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

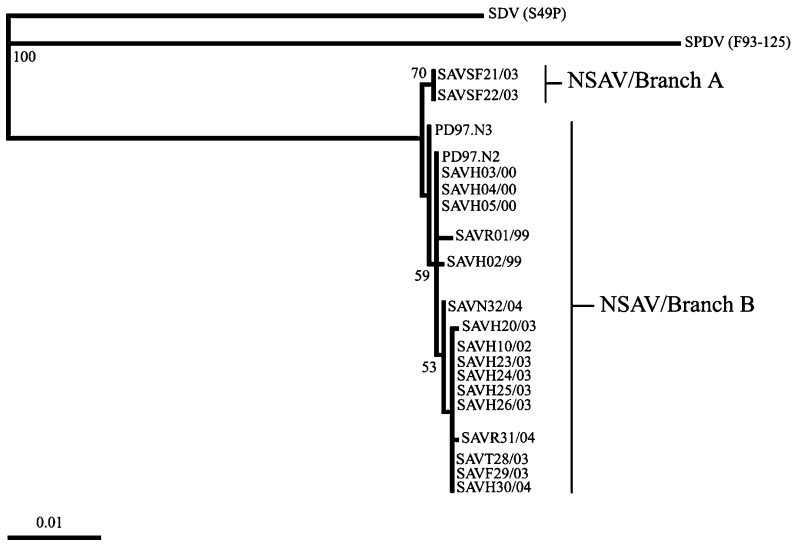

Fig. 2. Phylogenetic relationships among SAV isolates. SDV (S49P) and SPDV (F93-125) reference isolates are used as outgroups. The tree was bootstrapped using 1000 replicates, and bootstrap values <50 are not shown

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.

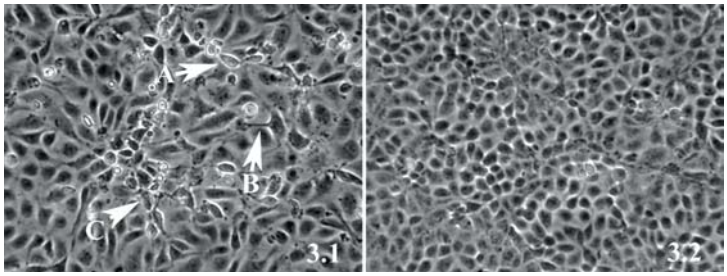

Fig. 3. CPE in CHSE-214 cells caused by NSAV infection. Figure 3.1 and 3.2 show cells after 13 passages of NSAV infection and uninfected control cells, respectively. NSAV CPE is recognized as curled up cells (**A**), pseudopodia-like extensions (**B**), and vacuolization in the cytoplasm (**C**)

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.

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Paper III

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Sensitive and specific detection of *Salmonid alphavirus* using real-time PCR (TaqMan®)

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Abstract

Pancreas disease is responsible for major economic losses in the European salmonid farming industry. It was previously believed that a single subtype (salmon pancreas disease virus) of the virus species *Salmonid alphavirus* (SAV) was responsible for all outbreaks of pancreas disease in the UK and Norway. However, the recent discovery that pancreas disease in Norway is caused by a new and distinct subtype of salmonid alphavirus, exclusively found in Norway, has advocated the need for better diagnostic tools. In the present paper, three real-time PCR assays for all known subtypes of salmonid alphavirus have been developed; the Q_nsP1 assay is a broad-spectrum one that detects RNA from all subtypes, the Q_SPDV assay specifically detects the salmon pancreas disease virus subtype, and the Q_NSAV assay only detects the new Norwegian salmonid alphavirus subtype.

The results demonstrated the assays to be highly sensitive and specific, detecting <0.1 $TCID_{50}$ of virus stocks. Regression analysis and standard curves calculated from the C_t-values from 10-fold serial dilutions of virus stocks showed that the assays were highly reproducible over a wide range of RNA input. Thirty-nine field samples were tested in triplicate and compared with traditional RT-PCR. Overall, the real-time assays detected 35 positive compared to 29 positives in standard RT-PCR, and were thus shown to be more sensitive for detecting salmonid alphaviruses in field samples.

Keywords: *Salmonid alphavirus*; Real-time PCR; Virus detection

1. Introduction

Pancreas disease in farmed salmonids is commonly associated with infections by *Salmonid alphavirus* (SAV). The disease occurs mainly in Atlantic salmon in their first or second year at sea, and diseased fish are often lethargic, with abnormal swimming behaviour. Histopathological lesions in association with pancreas disease always include various degrees of heart and skeletal muscle myopathy. Acute and chronic pancreatic lesions in exocrine pancreatic tissue may also be present in diseased fish (McLoughlin et al., 2002). The virus responsible for pancreas disease in Ireland and Scotland have been isolated and identified as an alphavirus, and the name salmon pancreas disease virus (SPDV) was suggested (Nelson et al., 1995; Welsh et al., 2000; Weston et al., 1999). Because pancreas disease affected fish from Norway show similar clinical symptoms and gross pathology, it has been of the common opinion that pancreas disease in the British Isles and Norway is caused by the same virus. However, Hodneland et al. (2005) recently showed that pancreas disease from Atlantic salmon and rainbow trout in Norway is in fact caused by a different and distinct virus subtype, and named it Norwegian salmonid alphavirus (NSAV). Together with the sleeping disease virus (SDV) (Boucher and Baudin Laurencin, 1994; Branson, 2002; Castric et al., 1997; Graham et al., 2003b; Villoing et al., 2000a) and salmon pancreas disease virus, the Norwegian salmonid alphavirus is included in the species *Salmonid alphavirus* in the genus Alphavirus of the family Togaviridae. All three salmonid alphavirus subtypes have a genomic organization characteristic to the Alphaviruses; with a positive-sense, single stranded genome of approximately 11.8 kb size. The 5′-terminal end codes for the four non-structural proteins (nsP1–nsP4) essential for virus replication, whereas the 3′-terminal comprises the genes for the structural proteins E1–E3,

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capsid and 6K. Nucleotide sequence comparisons have shown that Norwegian salmonid alphavirus, salmon pancreas disease virus and sleeping disease virus are approximately equal in evolutionary distance to each other, with differences ranging from 91.6 to 92.9% (Hodneland et al., 2005).

Previously, the diagnosis of pancreas disease and sleeping disease was based on clinical sign in combination with histopathological findings. Detection of antibodies or virus isolation in fish cells may also be used to verify the aetiology of the disease (Graham et al., 2003a; Jewhurst et al., 2004). However, the presence of virus-specific antibodies does not provide any information about the viraemic status of an infected fish, and considering the high percentage similarity of the structural proteins among the salmonid alphaviruses, the potential for cross-reactivity of serological assays is high. Furthermore, it is not possible to unequivocally distinguish subtypes of salmonid alphavirus in cell-cultures. As a result of the relative insensitivity of non-molecular detection methods, molecular methods such as RT-PCR based techniques have been developed for a number of fish RNA viruses, and have been demonstrated successfully to increase the detection rate. Villoing et al. (2000b) presented a two-step RT-PCR assay for detection of sleeping disease virus RNA in naturally infected salmonids, which also proved useful for amplification of salmon pancreas disease virus in experimentally infected fish. However, the RT-PCR could not discriminate between the two subtypes without further sequencing studies.

With the discovery of the third salmonid alphavirus subtype and the distinct geographical distribution of at least salmon pancreas disease virus and Norwegian salmonid alphavirus, the ability to distinguish between types or strains of virus that may have distinct biological properties is important for both national and international management and control of the disease. At present, existing methods are not sufficient to rapidly distinguish between the different pheno-/genotypes, and the development of a more powerful diagnostic assay for direct identification of salmonid alphavirus subtypes, with respect to sensitivity, specificity and speed will be useful. The real-time PCR technology is now used commonly for detection and quantification of many viruses (Mackay et al., 2002; Niesters, 2001; Niesters, 2002), however the only piscine viruses where real-time PCR assays have been developed are piscine nodavirus (Starkey et al., 2004), infectious salmon anemia virus (ISAV) (Munir and Kibenge, 2004) and infectious haematopoietic necrosis virus (IHNV) (Overturf et al., 2001).

The present paper describes the development and validation of real-time PCR assays for the sensitive detection and differentiation of three subtypes of salmonid alphavirus (salmon pancreas disease virus, sleeping disease virus and Norwegian salmonid alphavirus) using the TaqMan® probe chemistry. By developing these assays it is now possible to screen rapidly for all known salmonid alphavirus subtypes without the need for prior isolation and culture, or time-consuming post-PCR steps. The advantage of using real-time PCR for detection of salmonid alphaviruses not only saves time and labor, but also has the potential to differentiate and quantitate any subtype of salmonid alphavirus within the host.

2. Material and methods

2.1. Virus stocks and clinical samples

The specificity of the real-time PCR assay was determined using control strains as a RNA source. Cultured virus stocks from three different subtypes of salmonid alphavirus were used as reference templates in the real-time assays: Norwegian salmonid alphavirus isolated from Norwegian salmon suffering from pancreas disease (SavH10/02, Genebank accession no. AY604237), salmon pancreas disease virus from pancreas disease affected salmon from Ireland (F93-125, Genebank accession no: AJ316244), and a sleeping disease virus isolate originating from rainbow trout from France (kindly supplied by Dr. K.E. Christie, Intervet Norbio AS, Bergen, Norway). Virus titers ($TCID_{50}$) was $10^{5.8}$/ml for Norwegian salmonid alphavirus, $10^{7.6}$/ml for salmon pancreas disease virus, and $10^{6.25}$/ml for the sleeping disease virus cell culture. Heart tissues from 39 salmon from various fish farms were collected and tested to evaluate the performance of the real-time PCR assays from field samples. The fish in the salmon farms were diagnosed, or suspected to suffer from pancreatic disease.

2.2. RNA extraction

RNA extraction from both infected cell cultures and tissues was performed as described by Devold et al. (2000). The purity of the RNA was evaluated by measuring the absorbance ratio at 260/280 nm (optimal 1.8–2.0), and RNA quality was checked on ethidium bromide-stained agarose (1%) gel using UV illumination. RNA from tissue samples was dissolved in RNAse free water at a working concentration of 100 ng/ul.

2.3. Standard RT-PCR

Standard RT-PCR assays were performed by incubating 2 ul of dissolved total RNA with 1.0 ul (1 ug/ul) random hexamer pd(N)6 primer and 7.0 ul ddH_2O at 70 °C for 5 min and placed on ice. The RT-reaction was carried out at 37 °C for 60 min with 10 U Rnasin, 5.0 μl 5 × RT-buffer, 3.0 U M-MLV-reverse transcriptase, 1.25 μl DTT (200 mM), 2.5 μl dNTP (10 mM). The PCR was performed in a 25 ul reaction volume containing 2.0 μl cDNA template, 2.5 μl 10 × Taq buffer, 1.0 μl (10uM) of each PCR primer (Table 1), 2.0 μl (10 mM) dNTP mix, 0.1 μl (5 U/ul) Taq DNA polymerase and 16.4 μl ddH_2O. The PCR profile was as follows: one cycle at 95 °C in 3 min; then 40 cycles at 94 °C for 30 s; 55 °C for 45 s; and 72 °C for 90 s; followed by one cycle at 72 °C for 10 min. The amplification and cDNA synthesis were performed in GeneAmp PCR System 9700 (Perkin-Elmer). PCR products were visualized on an ethidium bromide-stained agarose (1%) gel using UV illumination.

2.4. Primers and probes

TaqMan PCR primers and probes were designed according to standard cycling conditions using the PrimerExpress software package (PE Applied Biosystems), and were derived from an

Table 1
Sequences and positions of primers and probes used for real-time assays and conventional RT-PCR

Oligonucleotide	Sequence	Amplicon length	Position	Position no.
Q_SPDV F primer	5′-ACAGTGAAATTCGACAAGAAATGC-3′	68	9555–9578	NC003930
R primer	5′-TGGGAGTCGCTGGTGAAAGT-3′		9603–9622	
Probe	FAM-5′-AGAGCGCTGACTCGGCAACCGT-3′-MGB		9580–9601	
Q_NSAV F primer	5′-CAGTGAAATTCGATAAGAAGTGCAA-3′	67	9431–9455	AY604235
R primer	5′-TGGGAGTCGCTGGTAAAGGT-3′		9478–9497	
Probe	FAM-5′-AGCGCTGCCCAAGCGACCG -3′-MGB		9457–9475	
Q_nsP1 F primer	5′-CCGGCCCTGAACCAGTT-3′	107	17–33	AY604235
R primer	5′-GTAGCCAAGTGGGAGAAAGCT-3′		54–69	
Probe	FAM-5′-CTGGCCACCACTTCGA-3′-MGB		103–123	
2234 F primer	5′-CGGGTGAAACATCTCTGCG-3′	539	9014–9032	AY604235
2767 R primer	5′-CTTGCCCTGGGTGATACTGG-3′		9533–9552	
F4 primer	5′-AGCGACTCCCAGACGTTTACG-3′	899	9487–9507	AY604235
R1 primer	5′-CGGTTTATCACTGCTTCGTACGA-3′		10363–10385	

alignment of available sleeping disease virus, salmon pancreas disease virus and Norwegian salmonid alphavirus sequences. Primer pairs and probes that demonstrated 100% homology to their respective sequences, and at the same time discriminated between the subtypes were selected (Fig. 1). Two of these real-time PCR assays, Q_SPDV and Q_NSAV, amplify an identical region in the E2 gene of salmon pancreas disease virus and Norwegian salmonid alphavirus, respectively. A common primer

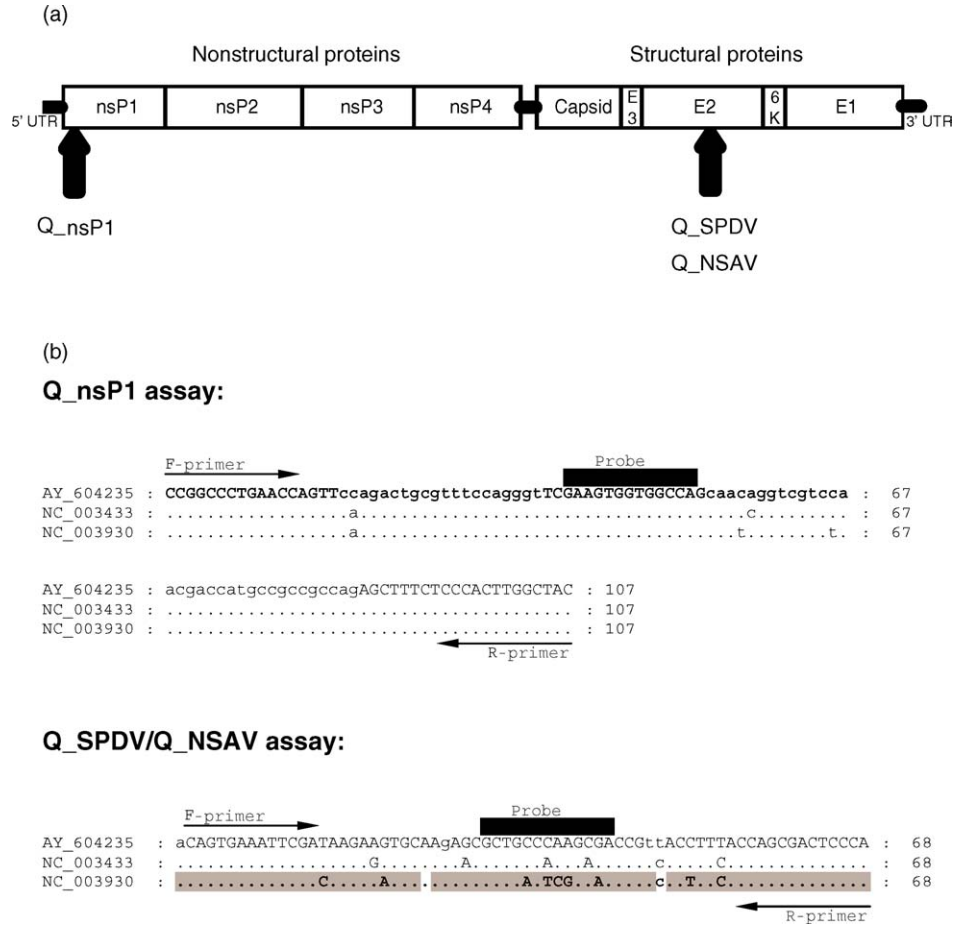

Fig. 1. (a) Orientation of primers and probes in relative position to the genome organization of salmonid alphaviruses. (b) Sequence comparisons of the three salmonid alphavirus subtypes, Norwegian salmonid alphavirus (AY604235), sleeping disease virus (NC003433) and salmon pancreas disease virus (NC003930) in the targeted regions for Q_nsP1, Q_SPDV and Q_NSAV assay. In the Q_nsP1 assay the common primers and probe for all salmonid alphaviruses are indicated by bold letters. Primers and probe used in the Q_SPDV assay are denoted by shaded, bold letters in the sequence for NC_003930. The Q_NSAV assay uses primers and probe sequences shown as letters in bold in the AY_604235 sequence.

pair and probe for all available salmonid alphavirus sequences was designed, and this real-time assay (Q_nsP1) amplifies a region in the 5′ end of the nsP1-gene. All primers and probes were obtained from PE Applied Biosystems. The PCR primers 2234F and 2767R were applied in the standard RT-PCR reaction, and amplify a 539 bp fragment of the E2 gene. This primer pair has been used previously and routinely for detection of salmonid alphaviruses in the laboratory. In addition, the primer pair F4-R1 (Hodneland et al., 2005) was used for amplification of infected tissue samples with subsequent sequencing in order to verify the identity of the virus species detected in the real-time assays. Details on all primers and probes used in this study are summarized in Table 1.

2.5. Real-time PCR

TaqMan assays were performed with 2 ul of cDNA (template), 900 nM of each primer, and 200 nM of probe in a total volume of 25 ul by using the TaqMan Universal PCR Master Mix w/AmpErase® UNG (PE Applied Biosystems). Amplification (45 cycles) and fluorescence detection were performed with the ABI Prism 7000 sequence detection system instrument as recommended by the manufacturer (PE Applied Biosystems). Each sample was tested in triplicate. Samples were considered positive when the fluorescence signal increased above the threshold cycle (C_t), and if the C_t value was $\leq$37.5. The threshold value for all tests was fixed at 0.2, which was within the linear phase of the exponential amplification and above background fluorescence noise. The cut-off C_t value at 37.5 was set based on two-fold dilutions (30 replicates) of a viral cDNA-stock, and denotes the mean C_t value in the highest dilution for which all 30 replicates were positive (results not shown).

2.6. Plasmid preparation

PCR fragments encompassing their respective probing regions of the Q_SPDV, Q_NSAV and Q_nsP1-assays for all three virus strains were cloned into the pCR®4-TOPO® vector (Invitrogen) under conditions recommended by the supplier. Plasmids were linearized and the template (dsDNA) copy number was calculated by UV spectroscopy at an optical density of 260 nm. Plasmid preparations were diluted to a final stock concentration of 5×10^7 copies/ul.

2.7. Standard curves and detection limits

For each real-time PCR assay a standard curve was generated using a 10-fold serial dilutions (three parallels) of the virus stock RNA's. An aliquot of 100 ul of each viral dilution was extracted as described above. Regression analysis, standard curve slopes s (C_t versus log quantity), and amplification efficiencies E ($E = [10^{1/(-\text{slope})}] - 1$) were calculated. If the amplification efficiency of the reaction is ideal, or 1, the PCR product concentration doubles during every cycle within the exponential phase of the reaction. The accepted amplification efficiency range is 0.9–1.1. A similar approach was used to make standard curves from salmonid alphavirus infected fish tissue (heart ventricle) from field samples (5-fold serial dilutions). This test was done in order to check for any limitations of the assays from field samples compared to virus samples from CHSE-214 cell cultures, such as the presence of endogenous inhibitors in template RNA preparations.

Data from the standard curve estimations above were also used to determine the limit of detection of the real-time assays. The detection limit describes the lowest amount of a template that can be detected under optimal conditions, and was defined as the highest dilution factor for which all samples (triplicate) were positive in the specific real-time assay. This was done by direct comparison of observed C_t values with the respective serial dilutions of infectious virus titers ($TCID_{50}$). Establishing the relationship between the real-time PCR detection limits and corresponding dilution of the $TCID_{50}$ virus stocks will give an indication of the detection performance of the two different methods. Similarly, detection limits for each real-time PCR assay was determined for serial dilutions of known amounts of linearized plasmid (template copies). This latter test was performed in order to check the efficiency of the PCR in the real-time assays.

3. Results

3.1. Specificity

The Q_nsP1 TaqMan assay was designed specifically to detect all known salmonid alphaviruses, whereas the Q_SPDV and Q_NSAV assays were designed to discriminate between salmon pancreas disease virus and Norwegian salmonid alphavirus, respectively. The location of primers and probes for all three assays were chosen carefully for distinguishing salmonid alphaviruses from other viruses. No fluorescent signal for the three assays was generated when RNA samples from tissues infected with ISAV, IPNV, and Nodavirus was tested (data not shown). The specificity of the different probes on salmonid alphavirus was assessed by testing the viral stocks of Norwegian salmonid alphavirus (Norway; SavH10/02), salmon pancreas disease virus (Ireland; F93–125), and sleeping disease virus (France). All three salmonid alphavirus strains were detected using the Q_nsP1 assay, whereas the Q_SPDV assay only detected the F93–125 samples and the Q_NSAV was positive for only for the SavH10/02 strain. Real-time PCR on all combinations of mixes of cDNA from the three virus strains did not affect the specificity of the different assays (data not shown). Furthermore, real-time PCR assays on the plasmid preparations did not produce any false negatives or positives. Standard RT_PCR on virus RNA using the F4-R1 primers produced amplicons of correct length, and subsequent sequencing of the PCR-products identified the samples as the correct virus species.

Total RNA preparations of heart tissue samples from 39 Atlantic salmon collected from various fish farms were screened (Table 2). The Q_nsP1 assay was first applied to check for presence of salmonid alphavirus in the samples. If the sample was found to be positive, the presence of salmon pancreatic disease virus or Norwegian salmonid alphavirus subtype were checked specifically by using the Q_SPDV and Q_NSAV real-

Table 2
Comparison of salmonid alphavirus detection from fish samples by real-time PCR assays and standard RT-PCR

Fish no.	Sample (location and year of collection)	TaqMan assay			Standard RT-PCR
		Q_nsP1	Q_SPDV	Q_NSAV	
1	HO (97)_1	31.40	–	32.69	3/3
2	HO (97)_2	36.20	–	37.74	0/3
3	HO (97)_3	–	–	–	0/3
4	HO (97)_4	–	–	–	0/3
5	HO (97)_5	–	–	–	0/3
6	HO (97)_6	29.70	–	31.19	3/3
7	HO (99)_1	24.90	–	26.39	3/3
8	HO (99)_2	30.10	–	31.63	3/3
9	SF (03)_1	32.02	–	33.50	3/3
10	SF (03)_2	32.30	–	33.72	3/3
11	SF (03)_3	31.60	–	33.12	3/3
12	SF (03)_4	29.22	–	30.62	3/3
13	SF (03)_5	19.77	–	21.33	3/3
14	SF (03)_6	33.59	–	35.20	1/3
15	SF (03)_7	34.87	–	36.33	0/3
16	SF (03)_8	28.88	–	30.36	3/3
17	SF (03)_9	35.10	–	36.68	0/3
18	SF(03)_10	34.19	–	35.69	3/3
19	HO (04)_1	31.39	–	32.82	3/3
20	HO (04)_2	27.19	–	28.65	3/3
21	HO (04)_3	23.63	–	25.10	3/3
22	HO (04)_4	19.80	–	21.23	3/3
23	HO (04)_5	33.37	–	34.91	2/3
24	HO (04)_6	28.63	–	30.13	3/3
25	HO (04)_7	23.25	–	24.59	3/3
26	HO (04)_8	19.27	–	20.72	3/3
27	HO (04)_9	22.60	–	24.11	3/3
28	HO (04)_10	30.01	–	31.61	3/3
29	HO (04)_11	33.03	–	34.55	2/3
30	SF (04)_1	–	–	–	0/3
31	SF (04)_2	33.32	–	34.32	2/3
32	SF (04)_3	37.49	–	39.01	0/3
33	SF (04)_4	36.81	–	38.33	0/3
34	SF (04)_5	33.89	–	35.48	1/3
35	SF (04)_6	31.64	–	33.01	3/3
36	SF (04)_7	29.09	–	30.55	3/3
37	SF (04)_8	35.41	–	36.99	0/3
38	SF (04)_9	30.18	–	32.01	3/3
39	SF (04)_10	34.81	–	36.33	2/3
No. of fish positive		35	0	35	29
No. of fish negative		4	39	4	10

time PCR. Thirty-five cases of Norwegian salmonid alphavirus were detected with both the Q_nsP1 and Q_NSAV assay. No cases with salmon pancreas disease virus from the samples were detected. Standard RT-PCR detected 29 salmonid alphavirus infected fish, all of these had C_t-values of <34.87 (Q_nsP1 assay). Some fish had very low C_t-values (<20.0) indicating large amount of viral templates in the examined tissue. The six fish which tested negative in the RT-PCR screening but were positive in the Q_nsP1 assay, had C_t-values of 36.20, 34.87, 35.10, 37.49, 36.81 and 35.41. Five of the 29 positive fishes were only positive in one or two of the replicates in the standard RT-PCR (Table 2). The C_t-values (Q_nsP1) from these five fishes ranged from 33.03 to 34.81. To validate the specificity of the TaqMan assays, cDNA from positive fish were checked with PCR with the F4–R1 primer combination, and sequencing showed that all were of the Norwegian salmonid alphavirus subtype.

3.2. Standard curves

The standard curve for the serial dilutions (10-fold) of the virus stocks were calculated for all three real-time PCR assays. The mean slopes for all assays were similar (Fig. 2 and Table 3), and the amplification efficiency (E) indicated near maximum PCR efficacy ($E = 0.965–1.035$). Almost identical standard curve slopes and C_t-values were obtained with dilutions either before RT at the RNA level, or at the cDNA level (data not shown). Furthermore, serial dilutions (5-fold) from infected tissues from field samples produced standard curve slopes and amplification efficiencies similar to the above (Table 3).

3.3. Sensitivity

Assay detection range for the three different real-time PCR assays and standard RT-PCR with the primer pair 2234F–2767R are summarized in Table 4. Each of the real-time assays was evaluated by comparison of the dilution limit C_t-value (C_t) and their respective virus titre. For the standard RT-PCR assay samples were considered positive when a visible band of the correct size was observed on an ethidium bromide-stained agarose gel. When tested on the salmon pancreas disease virus—virus stock the sensitivity of Q_nsP1 assay was slightly higher than the Q_SPDV assay, although not statistically significant. How-

Table 3
Summary of standard curve slopes (s), regression coefficients (R^2) and amplification efficiencies (E) for the three real-time PCR assays from viral stocks and fish tissue samples

Salmonid alphavirus subtype	Origin	Q_nsP1			Q_SPDV			Q_NSAV		
		s	R^2	E	s	R^2	E	s	R^2	E
Sleeping disease virus[a]	Viral stock	−3.22	0.999	1.004	–	–	–	–	–	
Salmon pancreas disease virus	Viral stock	−3.24	0.998	1.035	−3.37	0.999	0.980	–	–	–
	Fish sample	−3.30	0.990	1.009	−3.41	0.989	0.965	–	–	–
Norwegian salmonid alphavirus	Viral stock	−3.37	0.997	0.980	–	–	–	−3.31	0.996	1.005
	Fish sample	−3.39	0.991	0.972	–	–	–	−3.32	0.999	1.001

[a] Only virus samples from CHSE-214 cell cultures were tested.

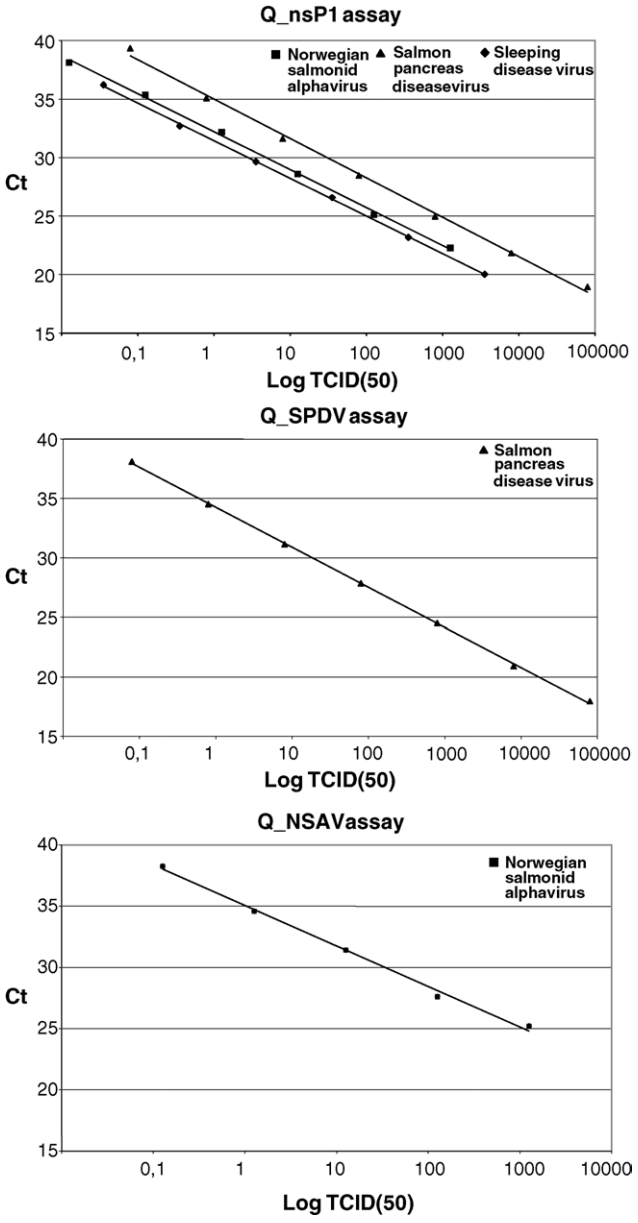

Fig. 2. Regression analysis and standard curves for the Q_nsP1, Q_SPDV and Q_NSAV assay from virus infected CHSE-214 cells supernatant. The starting amount (undiluted sample) for the virus stocks were $10^{5.8}$/ml, $10^{7.5}$/ml and $10^{6.25}$/ml for the Norwegian salmonid alphavirus, salmon pancreas disease virus and sleeping disease virus, respectively. Ten-fold serial dilutions of each virus stock were tested, and the mean C_t-values for each triplicate were plotted against the $TCID_{50}$ dilution. Regression analysis and standard curves for the Q_nsP1, Q_SPDV and Q_NSAV assay from virus infected CHSE-214 cells supernatant. The starting amount (undiluted sample) for the virus stocks were $10^{5.8}$/ml, $10^{7.5}$/ml and $10^{6.25}$/ml for the Norwegian salmonid alphavirus, salmon pancreas disease virus and sleeping disease virus, respectively. Ten-fold serial dilutions of each virus stock were tested, and the mean C_t-values for each triplicate were plotted against the $TCID_{50}$ dilution.

ever, the Q_NSAV assay was significantly less sensitive than the Q_nsP1 assay by an average of 2.7 C_t-values irrespective of the dilution factor. Amplification plots of the Q_nsP1 assay showed that dilutions of 10^{-6}, 10^{-5} and 10^{-6} for the salmon pancreas disease virus, sleeping disease virus, and Norwegian salmonid

Table 4
Sensitivity comparisons of the different real-time PCR assays and standard RT-PCR for the detection of salmonid alphaviruses

Dilution	Q_nsP1 assay						Q_SPDV assay		Q_NSAV assay		Standard RT-PCR*			
	Salmon pancreas disease virus		Sleeping disease virus		Norwegian salmonid alphavirus		Salmon pancreas disease virus		Norwegian salmonid alphavirus		Salmon pancreas disease virus		Sleeping disease virus	Norwegian salmonid alphavirus
	C_t	Result	C_t	Result	C_t	Result	C_t	Result	C_t	Result	Result		Result	Result
Undiluted	18.9	3/3	20.0	3/3	22.3	3/3	18.0	3/3	25.2	3/3	3/3		3/3	3/3
10^{-1}	21.8	3/3	23.2	3/3	25.1	3/3	20.9	3/3	27.6	3/3	3/3		3/3	3/3
10^{-2}	25.0	3/3	26.6	3/3	28.6	3/3	24.6	3/3	31.4	3/3	3/3		3/3	3/3
10^{-3}	28.5	3/3	29.6	3/3	32.2	3/3	27.9	3/3	34.6	3/3	3/3		3/3	3/3
10^{-4}	31.6	3/3	32.7	3/3	35/4	3/3	31.1	3/3	38.3	3/3	3/3		1/3	0/3
10^{-5}	35.1	3/3	36.2	3/3	38.1	3/3	34.5	3/3	–	0/3	0/3		0/3	0/3
10^{-6}	39.3	3/3	–	0/3	42.1	2/3	38.1	3/3	–	0/3	0/3		0/3	0/3
10^{-7}	–	0/3	–	0/3	–	0/3	–	0/3	–	0/3	0/3		0/3	0/3

The $TCID_{50}$ for undiluted virus samples were $10^{7.6}$/ml for salmon pancreas disease virus, $10^{6.25}$/ml for sleeping disease virus, and $10^{5.8}$/ml for the Norwegian salmonid alphavirus from cell culture.

Table 5
Sensitivity comparisons of the different real-time PCR assays on linearized plasmid preparations containing the appropriate target sequence

Plasmid copies	Q_nsP1 assay						Q_SPDV assay		Q_NSAV assay	
	SPDV_P1		SDV_P1		NSAV_P1		SPDV_P2		NSAV_P2	
	C_t	Result	C_t	Result	C_t	Result	C_t	Result	C_t	Result
10^8	17.42	3/3	16.95	3/3	17.36	3/3	17.12	3/3	19.84	3/3
10^7	20.67	3/3	19.02	3/3	20.42	3/3	20.25	3/3	22.76	3/3
10^6	23.92	3/3	22.30	3/3	22.74	3/3	23.09	3/3	25.51	3/3
10^5	27.25	3/3	26.17	3/3	26.24	3/3	26.84	3/3	29.27	3/3
10^4	31.10	3/3	29.07	3/3	29.79	3/3	30.39	3/3	32.28	3/3
10^3	34.41	3/3	32.26	3/3	33.21	3/3	33.67	3/3	35.69	3/3
10^2	37.26	3/3	35.86	3/3	37.01	3/3	37.09	3/3	39.30	3/3
10^1	39.97	3/3	39.49	3/3	39.55	3/3	39.72	3/3	42.56	2/3
1	41.99	2/3	42.13	1/3	41.12	1/3	43.79	1/3	–	0/3
Slope (s)	−3.29		−3.27		−3.25		−3.31		−3.25	

The plasmid copies refer to the total input of linearized plasmid copies in each reaction.

alphavirus strains, respectively, could be detected. Including all extraction steps, this correlates with a sensitivity of 0.08 $TCID_{50}$ for salmon pancreas disease virus, 0.04 $TCID_{50}$ for sleeping disease virus and 0.01 for Norwegian salmonid alphavirus per reaction.

Similarly, the real-time PCR on the linearized plasmid dilution series also indicated that the Q_NSAV assays was less sensitive than the Q_nsP1 assay. Using the same plasmid dilutions as template there was an average of 2.6 C_t-values higher for the Q_NSAV assay compared with the C_t values for the Q_nsP1 assay.

Overall, the detection limit for Q_NSAV was between 10 and 100 plasmid copies (NSAV_P2), and the Q_SPDV assay detected 1–10 copies of the SPDV_P2 plasmid. Sensitivity test of the Q_nsP1 assay on the plasmids SPDV_P1, SDV_P1 and NSAV_P1 showed that between 1 and 10 plasmid copies could be detected for all three plasmid preparations (Table 5). Regression analysis on the plasmid dilution series demonstrated that all assays were quantitative with standard curve slopes (s) ranging from −3.25 to −3.31.

4. Discussion

Initially, a real-time PCR assay (Q_SPDV) was designed as a less labour consuming and more reliable diagnostic test than previous assays used by the official diagnostic laboratory. Primers and probes were designed to match 100% to the available salmon pancreas disease virus sequences deposited in the Genebank (NC003930, AJ012631). The Q_SPDV assay was tested on a laboratory strain of pancreas disease virus with great success, but when applied on field samples from diseased fish diagnosed as pancreas disease the assay were not able to produce any positive results. Through a series of RT-PCRs and sequencing studies it became clear that the virus causing pancreas disease in Norway is a separate subtype of the salmonid alphavirus species (Hodneland et al., 2005), and that the sequence differences between Norwegian salmonid alphavirus and salmon pancreas disease virus in the targeted Q_SPDV area were sufficiently large to effectively prevent any amplification from Norwegian salmonid alphavirus templates. A new assay was designed which amplifies the same region as in the Q_SPDV assay, but directed specifically against the Norwegian salmonid alphavirus sequence (AY604235). Furthermore, a common assay (Q_nsP1) specific for salmonid alphavirus was made in order to detect any of the three subtypes of the salmonid alphavirus species (salmon pancreas disease virus, Norwegian salmonid alphavirus and sleeping disease virus). In the present study, it is shown that the specificity for the three assays is 100% for all available salmonid alphavirus sequences, and gives no false positives or negatives. The use of AmpErase uracil-*N*-glycosylase (UNG) in the PCR amplification step also greatly reduces or eliminates the potential source of carry-over contamination of samples which is crucial, at least for diagnostic purposes, in laboratories with large sample through-put (Burkardt, 2000; Hofmann-Lehmann et al., 2000; Pennings et al., 2001; Taggart et al., 2002).

A wide range of virus concentrations was linearly amplified in the Q_nsP1, Q_SPDV and Q-NSAV assays, with a near maximum PCR efficiency for all three (Table 3, Fig. 2). Similarly, plasmid copies ranging from 10^8 to 10 per reaction tested positive with the assays with amplification efficiencies (*E*-values) near 1.0. The common Q_nsP1 assay developed for all salmonid alphavirus species detected virus concentrations ($TCID_{50}$) from serial dilutions of virus stocks ranging from 10^{49}/reaction for salmon pancreas disease virus, to 0.01/reaction for the Norwegian salmonid alphavirus virus stock. The observed linearity in the wide range of virus titres should allow detection of salmonid alphaviruses from field samples in all stages of the disease in fish; early infection, viremiae with clinical pancreatic disease, or latency/persitent infections.

The Q_SPDV assay was equally sensitive as the Q_nsP1 assay when tested on the same template dilution series, making both assays suitable for further applications on studies on the salmon pancreas disease virus subtype. However, the Q_NSAV assay performed consistently lower than the Q_nsP1 assay when both assays were tested on identical template. This is somewhat surprising since the Q_NSAV assay detects and amplifies the same target region as in the Q_SPDV assay. Amplification and sequencing of this particular Norwegian salmonid alphavirus

genome stretch shows that the Q_NSAV primer and probes match 100% to the Norwegian salmonid alphavirus genome, and that mismatches cannot account for the lowered sensitivity of Q_NSAV. The differences between the primers and probes for the Q_SPDV and Q_NSAV are shown in Fig. 1(b), and involve two substitutions in both the forward and reverse primer, and five substitutions in the probe sequence. These substitutions do not alter the G + C composition or introduce any hairpin structures or stretches of identical nucleotides in the primers and probes. However, although not obvious, one or several of these substitutions must account for the significantly lower sensitivity in the Q_NSAV assay compared with Q_nsP1. The Q_NSAV assay is thus not an optimal real-time PCR detection for Norwegian salmonid alphavirus under the conditions used herein, demonstrating that ideally more that one assay should be tested for a target template to ensure that the real-time PCR is sufficiently sensitive for its purpose.

The Q_nsP1 assay was 10–100-fold more sensitive than standard RT-PCR on all virus stocks. As a consequence of the increased sensitivity of the real-time PCR assays the number of positive field samples also increased. The Q_nsP1 and Q_NSAV assays were able to detect target RNA in six samples which were negative with standard RT-PCR. These six fish had high C_t-values and probably reflect either a carrier status of pancreas disease, pre-viraemia, or post-viraemia where the host is in the process of clearing the virus. The same pattern with increased sensitivity was observed with a real-time assay for detection of Infectious salmon anemia virus (ISAV) (Munir and Kibenge, 2004). They showed that all fish that were positive in the real-time assay, but negative in the corresponding conventional RT-PCR, had C_t-values 36 or higher, and proposed that the increased sensitivity in their real-time PCR assay would be useful for detecting subclinical ISAV infections. Preliminary data (personal observation) have shown that the Norwegian salmonid alphavirus resides in the infected fish in low, but stable numbers for at least 6.5 months after the initial infection, indicating that replication of Norwegian salmonid alphavirus occur in surviving fish after the viraemic phase. In view of the short production cycle period in seawater, salmon could thus be considered a lifelong carrier of the virus. Interestingly, the lowest C_t-values from the Q_nsP1 assay on field samples (Table 2) are approximately the same as for the undiluted virus stocks. This indicates an extensive virus replication in the heart tissue of fish in a viraemic phase, although this cannot be verified without an adequate normalization of the above data.

The true amount of infectious virus particles is not necessarily detected by a real-time assay. It is likely that such an assay will over-estimate the number of infectious particles since viral RNA-target also is detected from replication intermediates, unpacked genomes, defective particles and free viral RNA from damaged cells. In the present study, the relationship between C_t-values from the real-time PCR assays and the $TCID_{50}$ from the viral stock dilution series was investigated. As expected, the viral RNA detected from dilutions corresponding to $TCID_{50}$ lower than 1.0. As little as 0.08 $TCID_{50}$ for salmon pancreas disease virus, 0.04 $TCID_{50}$ for sleeping disease virus and 0.01 for Norwegian salmonid alphavirus were detected, which suggests that a considerable amount of target RNA from the cell-cultures originate from non-infectious virus particles, or that the $TCID_{50}$ assays have low sensitivity.

From the plasmid dilution series it was demonstrated that the Q_nsP1 assay detects as few as 1-10 copies of linearized plasmid (ds DNA) containing the target sequence. Together with the observed slope values close to −3.34, this indicates that the PCR amplification in the assay is near optimal and is a very good candidate for use in absolute quantitation. The uncertainty of applying the Q_nsP1 assay in absolute quantitation of salmonid alphavirus target thus rests in the efficiency of the cDNA synthesis. As pointed out by Bustin and Nolan (2004) and Stahlberg et al. (2004), the efficiency of the reverse transcriptase step depends on the priming strategy, amount of RNA input, and choice of reverse transcriptase enzyme, which all varies among different genes. A validation of the Q_nsP1 assay for possible use in absolute quantification should first be clarified through serial dilutions of known numbers of salmonid alphavirus RNA-templates, with subsequent cDNA synthesis and real-time PCR. In most cases, however, the need for absolute quantification of the virus is not required, and it is sufficient to merely document the relative changes of salmonid alphavirus templates between varying experimental conditions (Bustin, 2000; Mackay et al., 2002; Pfaffl, 2001). This can be achieved by monitoring simultaneously a non-regulated reference target; either internal or added externally.

A real-time PCR technique for routine detection of all subtypes of salmonid alphavirus has been developed (Q_nsP1-assay), and has further potential use a quantitative tool in field or experimental studies of salmonid alphaviruses. The method is rapid, sensitive and specific, without amplifying product from other piscine viruses occurring in the salmon industry. Further optimization of the real-time assays could result in a reliable multiplex assay to specifically detect any of the three subtypes of salmonid alphavirus in the same reaction. The most significant advantages of real-time over conventional diagnostics of pancreas disease are time and labour savings. Where the traditional diagnosis of pancreas disease usually takes minimum one (standard RT-PCR) or several days (histopathology and/or cell culturing), the real-time PCR assay reported here can provide a reliable confirmation of salmonid alphavirus within 5 h. Additional time-savings can be achieved by using a commercial available one-step real-time RT-PCR, and thereby exclude the need for a separate cDNA synthesis step. The Norwegian salmonid alphavirus appears to be the only salmonid alphavirus subtype occurring in Norway, and is at present not reported elsewhere (present study; Hodneland et al., 2005). The salmon pancreas disease virus subtype is restricted to the UK, whereas the sleeping disease virus is reported from both France and UK (Branson, 2002). By using the above real-time PCR asssays it is now possible to easily distinguish between the subtypes of salmonid alphavirus, which is important for possible prevention of introduction of a specific subtype into new countries or areas where this subtype has not been reported previously. Also, in countries where a specific salmonid alphavirus is naturally occurring, early detection and confirmation of a suspected salmonid alphavirus aetiology of diseased salmonids is important for in-farm man-

agement, and may provide valuable information to slow down or stop the spread of the disease to new locations or regions.

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