Characterization of Salmonid alphavirus subtype 3

Recombination and adaptation

Philosophiae doctor (PhD) Thesis

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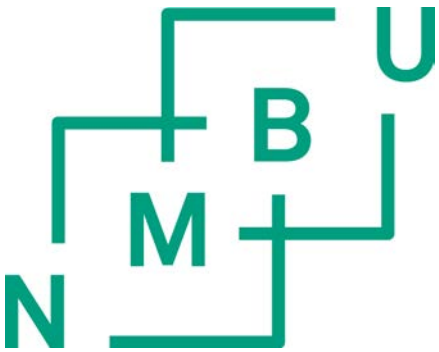

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Oslo, June 2016

Elin Petterson

SUMMARY

Pancreas disease (PD) affecting Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss)* is a major burden in European salmonid aquaculture and causes major economical losses every year. The disease is caused by Salmon Pancreas disease virus (SPDV), also referred to as salmonid alphavirus (SAV), which belongs to the genus alphavirus within the family *Togaviridae*.

Six subtypes of SAV have so far been reported where SAV subtype 3 and a marine variant of subtype 2 is found in Norway. Currently one commercial vaccine is available, but the effect under field conditions have been debated. Documentation of virulence characteristics and field oriented genome data have been scarce and this work was initiated to enlighten these subjects. Using a SAV3 isolate cultured in both CHSE and AGK cell line, the thesis shows that adaptation to AGK cells results in an isolate with a higher replication efficiency and higher virulence *in vitro*, compared to CHSE-adapted earlier passages. However, when tested for *in vivo* virulence in Atlantic salmon the results was reversed. Full-length genome sequencing revealed distinct differences between the different adapted passages.

Full-length genome sequences of SAV3 strains obtained from heart tissues collected from PD outbreaks spread along the Norwegian coastline confirmed high sequence identity within SAV3 strains, with a mean nucleotide diversity of 0.11 %. These samples, obtained directly from heart tissue without propagation in cell culture, include defective viral RNA with numerous genome deletions of varying size. Deletions in the RNA occurred in all virus strains and were not distributed randomly throughout the genome but tended to aggregate in certain areas/domains of the genome. This work was followed by experimental documentation of SAV3 RNA recombination *in vivo* where Atlantic salmon were injected with a combination of a SAV3 6K-gene deleted cDNA plasmid, encoding a non-viable variant of SAV3, and a helper cDNA plasmid encoding structural proteins and 6K only. A recombinant virus was grown from plasmid-injected fish, shown to infect and cause pathology in salmon after experimental exposure. In addition, imprecise recombination created RNA

deletion variants in fish that were co-injected with the two cDNA plasmids and the deletion genome variants were similar to what was found from field infections. Prediction of the RNA secondary structure indicated that such deletions are initiated at loops of unpaired nucleotides.

To summarize, SAV3 adapts to cell culture and in the search for virulence motifs *in vivo* guiding is important. The documentation of SAV3 RNA recombination is the first experimental documentation of alphavirus recombination in an animal and gives new insight into the formation of defective virus variants.

SAMMENDRAG

Salmon Pancreas disease virus (SPDV), også kalt salmonid alphavirus (SAV), er årsaken til pankreas sjukdom (PD) hos atlantisk laks (*Salmo salar* L.) og regnbueørret (*Oncorhynchus mykiss).* Sykdommen er et stort problem i europeisk akvakultur og forårsaker store økonomiske tap hvert år. SAV er et alphavirus i *Togaviridae* familien. SAV har per i dag 6 kjente subtyper, der SAV subtype 3 og en marin variant av subtype 2 finnes i Norge. En kommersiell vaksine mot sykdommen er tilgjengelig, men effekten av den har vært diskutert. Virulensegenskaper og sekvensering av feltisolater har i liten grad vært gjennomført og i denne studien blir disse temaene studert.

Ved å bruke et SAV3 isolat, dyrket i både CHSE og AGK celler, viser resultatene at viruset adapteres til celle kulturen benyttet. Ved adaptasjon til AGK celler får man et virus som replikerer mer og affiserer celle viabilitet negativt, i tillegg til å gi økt cytopatogen effekt sammenliknet med de CHSE adapterte passasjene. I et eksperimentelt fiskeforsøk *in vivo*, var effekten av *in vitro* celle adaptasjon reversert. Full lengde sekvensering av virus genomet viste sekvensforskjeller mellom de adapterte virusene.

Prøver fra laks, fra anlegg med PD diagnose spredt langs norskekysten, ble analysert og SAV3 genomene full lengde sekvensert. Sekvensene var fra virus infisert hjerte vev, for å unngå påvirkningen av celle kultur adaptasjon. Analysene viste høy grad av likhet mellom SAV3 variantene, med en gjennomsnittlig nukleotid diversitet på 0.11%. I tillegg viste studien at under infeksjon med SAV3 i felt genereres tallrike defekte virus RNA, i form av genom delesjoner. Delesjonene forekom i alle virus og viste tendenser til aggregering i enkelte områder.

Dette arbeidet ble fulgt opp av dokumentasjon på SAV3 RNA rekombinering *in vivo* i fisk. Atlantisk laks ble injisert med et SAV3 6K-gen deletert cDNA plasmid, som kodet for en ikke levedyktig variant av SAV3, sammen med et hjelpe cDNA plasmid som kodet kun for de strukturelle proteinene og 6K. Det rekombinerte viruset ble dyrket fra plasmid injisert fisk og infiserte og forårsaket patologi i laks. I tillegg ble upresis rekombinering bekreftet i form av RNA delesjons varianter i fisk injisert med cDNA plasmid. Delesjonene var i overenstemmelse med funnene av RNA delesjoner i forrige studie, fra felt

infeksjoner. Prediksjon av sekundær strukturen til SAV3 RNA indikerer at slike delesjoner blir initiert i områder av uparede basepar.

For å oppsummere; SAV3 adapteres til celle kultur og i kartleggingen av virusets virulens egenskaper er det viktig med *in vivo* basert bekreftelse og kunnskap. Dokumentasjonen av SAV3 RNA rekombinering er de første eksperimentelle data av alphavirus rekombinering i et dyr og gir ny innsikt til dannelsen av defekte virus RNA.

LIST OF PAPERS

Paper I

Natural infection of Atlantic salmon (*Salmo salar* L.) with salmonid alphavirus 3 generates numerous viral deletion mutants

Authors: Petterson E, Stormoen M, Evensen Ø, Mikalsen AB, Haugland Ø

Published: *Journal of General Virology 2013, 94, 1945–1954*

Paper II

In vitro adaptation of SAV3 in cell culture correlates with reduced *in vivo* replication capacity and virulence to Atlantic salmon (*Salmo salar* L.) parr

Authors: Petterson E, Guo TC, Evensen Ø, Haugland Ø, Mikalsen AB

Published: *Journal of General Virology 2015, 96, 3023–3034*

Paper III

Experimental piscine alphavirus RNA recombination *in vivo* yields both viable virus and defective viral RNA

Authors: Petterson E, Guo TC, Evensen Ø, Mikalsen AB

Published: Scientific Reports; *submitted*

ABBREVIATIONS

PD	Pancreas disease
SPDV	Salmon pancreas disease virus
SAV	Salmonid alphavirus
CHSE	Chinook salmon embryo cells
AGK	Asian Grouper skin cells
ISAV	Infectious salmon anemia virus
IPNV	Infectious pancreas necrosis virus
PMCV	Piscine myocarditis virus
PRV	Piscine reovirus
CMS	Cardiomyopathy syndrome
CPE	Cytopathic effect
CHH-1	Chum salmon heart cells
ER	Endoplasmic reticulum
HSMI	Heart and skeletal muscle inflammation
NFSA	Norwegian Food Safety Authorities
NSAV	Norwegian salmonid alphavirus
SESV	Southern elephant seal virus
SFI	Semliki Forest virus
SINV	Sindbis virus
CHIKV	Chikungunya virus
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus
EEEV	Eastern equine encephalitis virus
ORF	Open reading frame
UTR	Untranslated region
nsP	Non-structural protein
CSE	Conserved sequence element
DI RNA	Defective interfering RNA
DIP	Defective interfering particles
IFN	Interferon
MOI	Multiplicity of infection
VAPP	Vaccine-associated paralytic poliomyelitis
RRV	Ross river virus

INTRODUCTION

General background

The Norwegian aquaculture industry has grown to become an industry of major importance to the Norwegian economy and to the communities along the coast. Commercial salmon farming started around 1970 and today fish farms are located all along the Norwegian coast from south to north. Over decades the production has been growing and the production has doubled since 2005. Atlantic salmon and rainbow trout constitutes 99 percent of the production. 1.035.000 tons of salmon with a value of 47.7 billion NOK was exported in 2015, which is a 3.7 per cent increase from the year before (1). The massive increase and in general high production have resulted in a range of challenges related to environmental sustainability of the industry. The government has identified five key areas with potentially negative impact on the environment: diseases and parasites, escaped fish/genetic interaction, pollution and discharges, use of coastal areas and feed and feed resources (2). Diseases and parasites are of environmental concern because of the risk to the marine environment, but also responsible for large economical losses for the industry. Commercially available vaccines are mandatory and are highly protective against several important bacterial fish pathogens (3, 4), but viral diseases still remain a significant challenge. The most important viral diseases in Norwegian salmon farming are listed in Table 1 below.

Table 1. Overview of the most important viral infections in farmed Atlantic salmon in Norway 2015 (not in order of importance)

Virus	**Disease**
Infectious salmon anemia virus (ISAV)	Infectious salmon anemia (ISA)
Infectious pancreas necrosis virus (IPNV)	Infectious pancreas necrosis (IPN)
Salmon pancreas disease virus/Salmonid alphavirus (SAV)	Pancreas disease (PD)
Piscine myocarditis virus (PMCV)	Cardiomyopathy syndrome (CMS)
Piscine reovirus (PRV)	Heart and skeletal muscle inflammation (HSMI)

Among these, infections with SPDV, PMCV and PRV cause lesions in the heart and might have similar clinical appearance with inflammation and cardiomyocytic necrosis. An exact diagnosis can be determined by histopathological examination of the heart and other target organs combined, i.e. pathological changes in pancreas combined with necrosis and myositis of the red/white skeletal muscle in PD, heart changes combined with red skeletal muscle myositis (only) in HSMI, and spongious necrotizing myocarditis in CMS are used to differentiate the three diseases. Vaccines are commercially available for IPN, ISA and PD, but the protection offered is debatable.

Pancreas disease

Pancreas disease (PD) is a contagious disease in salmonid fish caused by salmonid pancreas disease virus (SPDV), also referred to as salmonid alphavirus (SAV). The initial descriptions of PD originate from the late 1970s and early 1980s in Irish and Scottish Atlantic salmon farms (5). In Norway the first reports of the disease came in the late 1980s (6) and has more or less been increasing since (Figure 1). In 2007 PD became a national notifiable disease (list 3) and the Norwegian Food Safety Authorities (NFSA) established national regulations in order to limit the spread of the disease.

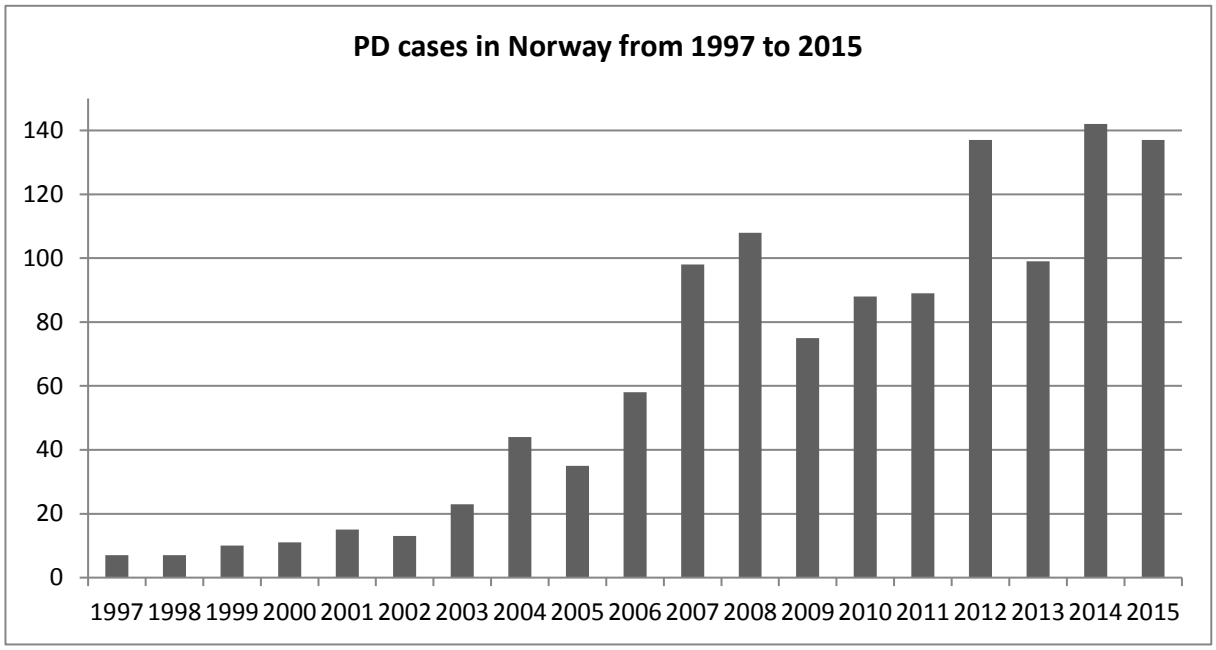

Figure 1. PD cases from 1997 to 2015 reported by the Norwegian Veterinary Institute. Sites diagnosed with PD and sites with suspicion of PD are included, both from salmon and rainbow trout. From 2012 PD caused by the marine SAV2 are included. Subtype identification is not performed routinely. For 2016 (not shown), the numbers are very high (52) by end of May (source: National Veterinary Institute).

Salmonid alphavirus subtype 3 (SAV3) was the only subtype found in Norway until 2011 when PD caused by a marine salmonid alphavirus subtype 2 (SAV2) was discovered for the first time in a farm growing Atlantic salmon (7), located in mid-Norway (Figure 2).

Pancreas disease leads to a prolonged loss of appetite, growth retardation and reduced filet quality. The economic loss for the industry is therefore large due to extended production time to slaughter and waste of feed. Diseased fish show degeneration and necrosis of acinar pancreatic tissue, cardiomyocitis and subsequent skeletal muscle degeneration and inflammation (8, 9). Mortality can occur but is generally low for both SAV3 and SAV2, although with a wide range.

Salmon pancreas disease virus

Historically, nomenclature of infections caused by salmonid pancreas disease virus has been complex. A spherical virus which morphologically resembled members of the *Togaviridae* was first isolated from an outbreak of PD in Atlantic salmon in Ireland (in 1994). This isolate was officially named salmon pancreas disease virus (SPDV), but have later been referred to as salmonid alphavirus subtype 1 (SAV1) (10) and was the first alphavirus reported in fish (11). However, a disease of rainbow trout held in freshwater in France was, despite sharing pathology, named sleeping disease due to the behavior of infected fish ("sleeping" at the bottom of the tanks). Isolation of the virus was reported by Castric in 1997 (12) and named in accordance with disease name as sleeping disease virus (SDV). This was subsequently sequenced and characterized as an alphavirus closely related to SPDV and has later also been referred to as SAV2 (13). By comparison, SPDV and SDV were found to be very similar at the genetic level, cross-reacted serologically and experimental infections in Atlantic salmon and rainbow trout confirmed that disease lesions induced were similar by histopathological examination (14). Weston et al. (2002) concluded that SPDV and SDV are closely related isolates of the same virus species suggesting the name salmonid alphavirus. In 2005 the alphavirus causing PD in Norway was characterized and revealed that Norwegian isolates are genetically different from the first SPDV and SDV isolates, and suggested as a separate subtype, Norwegian salmonid alphavirus (NSAV, later referred to as SAV3) (15). At the same time Weston et al. (2005) proposed that salmonid alphaviruses should be assigned to three genetically different subtypes (SAV1-3) based on nucleotide sequence criteria solely and not being referred to as either SPDV or SDV. A comprehensive study on the variation of salmonid alphaviruses analyzing 48 virus isolates from Ireland, Scotland, Norway, France, Italy, England, Spain and Northern Ireland were performed based on partial sequence data from nsP3 and E2 and proposed three more subtypes of salmonid alphavirus splitting SAV1 into SAV1 and SAV4-6 (15, 16). The authors also reported strains from marine production of Atlantic salmon clustering with freshwater isolates of SAV2 (15, 16). The distribution of the subtypes in Europe are illustrated in Figure 2. A comparative experimental study was conducted in Atlantic salmon in a fresh water cohabitation

trial, and showed that all subtypes (SAV1-6) caused pathological changes typical of pancreas disease, although relative virulence of the strains varied (17).

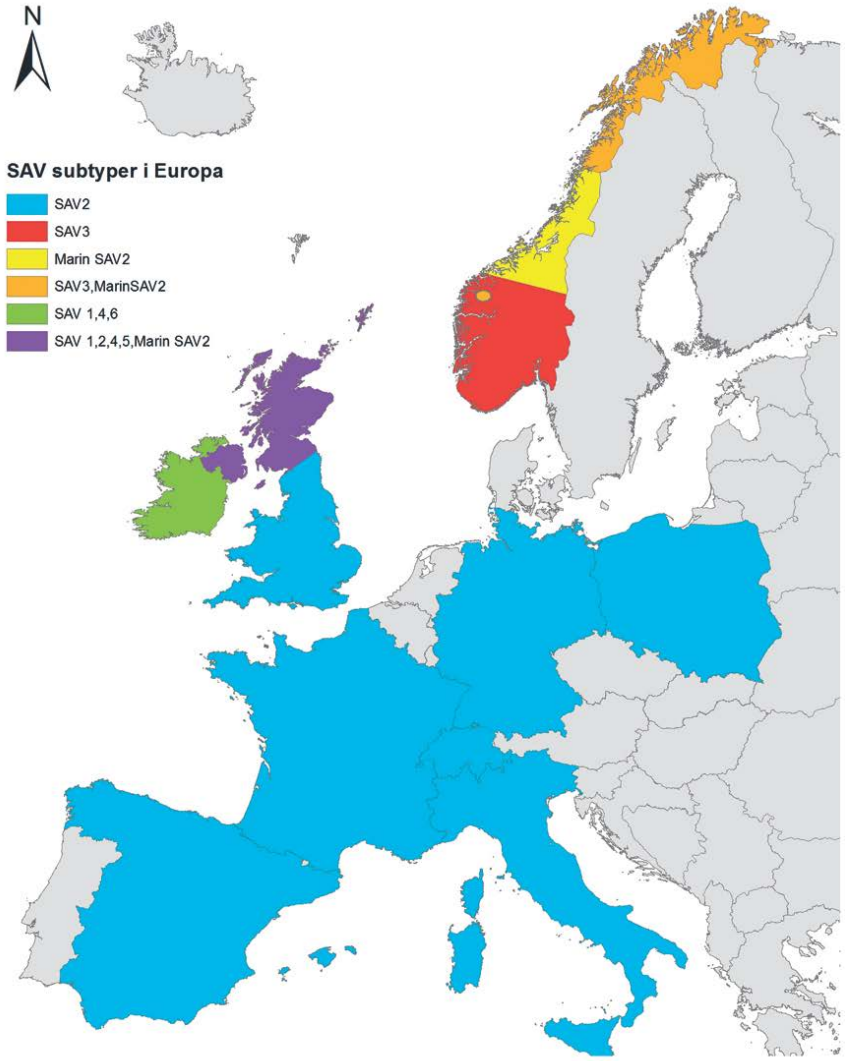

Figure 2. Geographical orientation of SAV subtypes in Europe (Source: Norwegian Veterinary Institute (18), reprinted with permission).

Alphavirus

Salmonid alphavirus is a positive-sense single-stranded RNA virus of the family *Togaviridae*, genus *Alphavirus.* Alphaviruses infect a broad range of insects and vertebrate hosts and the virus survives in nature by alternately replication in a vertebrate host and a hematophagous arthropod. Alphaviruses that cause disease in humans are arthropod-borne viruses (arboviruses) and are transmitted by mosquitos. They replicate and cause a persistent infection in the salivary glands of the arthropod (19) and are transmitted to the vertebrate host through the bite. The aquatic alphaviruses, SAV and Southern elephant seal virus (SESV), can be transmitted without an insect vector (20) but the presence of the virus within salmon lice, makes it unclear whether lice contribute to the infection either directly or indirectly (21-23).

Alphaviruses are a diverse group and have been isolated from all continents except Antarctica. Currently 31 alphavirus species are recognized based on genetic distance and ecological niche (International Committee on Taxonomy of Viruses, 2013). Alphaviruses are commonly referred to as `Old World` and `New World` viruses, roughly reflecting their geographical distribution (24). Old World viruses (Africa, Europe and Asia) are generally associated with rheumatic disease in humans where Semliki Forest virus (SFV), Sindbis virus (SINV) and Chikungunya virus (CHIKV) are the most studied prototypes (25). The New World viruses, which include Venezuelan, Eastern and Western Equine Encephalitis viruses (VEEV, EEEV and WEEV) are located in the Americas and primarily associated with potentially fatal encephalitic disease (26-28). SAV is the most divergent alphavirus with regard to genetic distance and phenotypical characteristics, and is the only alphavirus with fish as a host (29, 30).

Virus structure and entry

Alphaviruses are small membrane enveloped virions of 65-70nm in diameter. The membrane includes structural glycoproteins E1, E2 and E3 and enclose an icosahedral virion made of numerous copies of a capsid protein (Figure 4). The virus contain a single stranded positive–sensed RNA genome with general genomic structure conserved among all alphaviruses (31). The genome is approximately 12 kilobases long, consisting of two open reading frames (ORFs) (19) and three untranslated regions (UTRs) at 5´-and 3´-end in addition to an internal untranslated region between the ORFs (Figure 3) (32-34). The RNA is capped at the 5´ end and polyadenylated at 3´ end. The first ORF covers approximately two thirds of the genome and encodes the replicase polyprotein which after translation are cleaved into four non-structural proteins (nsP1-4). The second ORF encodes the structural proteins and is initially translated as a polyprotein precursor which are cleaved into capsid, E3, E2, 6K and E1 protein. Alphaviruses contain four conserved sequence elements (CSEs), meaning sequence structures that are conserved among alphaviruses and important for replication. They function as promotors for transcription by the viral RNA polymerase and are located at 5´UTR (CSE1), in nsP1 (CSE2), in the internal UTR (CSE3) and in the 3´UTR (CSE4). The CSE3 is the promotor for transcription of the subgenomic mRNA that contains the structural ORF (19).

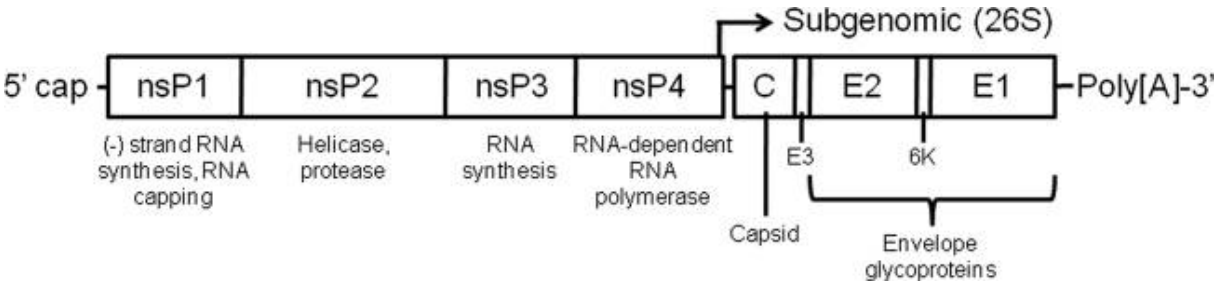

Figure 3. Illustration of the alphavirus genome showing the 5´cap, 5´untranslated region, nonstructural polyprotein open reading frame (ORF1) and major functions of the individual proteins, subgenomic promotor, structural polyprotein open reading frame (ORF2), 3´untranslated region and poly (A) tail. Reprinted with permission from publisher American Society for Microbiology (ASM).

The lipid bilayer covering the nucleocapsid usually contains the two surface glycoproteins E1 and E2, constituting 80 trimers of an E1/E2 heterodimer. The glycoproteins mediate attachment, fusion and penetration of the host cell. The E2 protein is responsible for receptor binding with a possible interaction with E1. One or several host receptors may be involved and the virus particle is taken up by endocytosis (19, 35). The pH in the endosome drops and triggers fusion of viral membrane with endosomal membrane. The nucleocapsid is released into the cytoplasm and cellular ribosomes finalizes the uncoating, and viral RNA is released for the initial translation (36) (Figure 5).

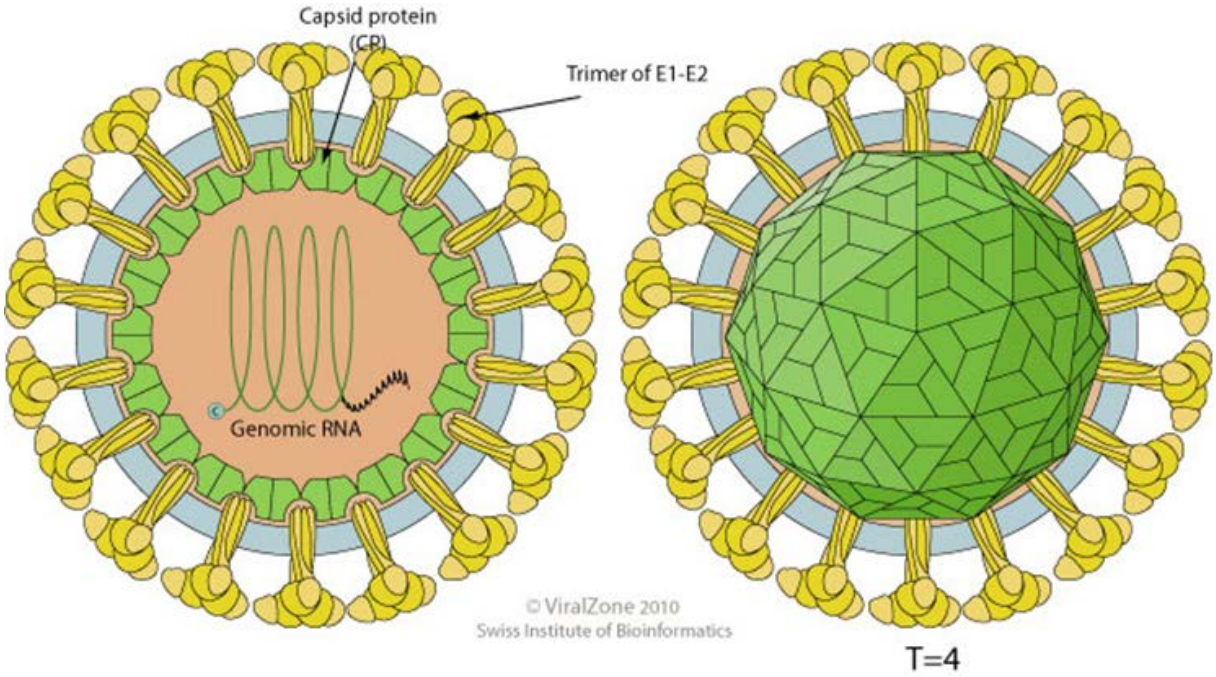

Figure 4. Alphavirus virion. Enveloped, icosahedral nuclecapsid, 65-70nm in diameter. The envelope contains 80 spikes, each spike are a trimer of E1/E2 proteins. Printed with permission from the Swiss Institute of Bioinformatics.

Replication

After the release of viral RNA the genome serves as messenger RNA for the synthesis of the non-structural or replication proteins (Figure 5). It also serves as a template for a complementary minus-

strand RNA. The translation of the first ORF results in polyprotein P1234, which is further processed into nsP1-4. The initial cleavage results in an early replication complex P123 and nsP4 where the complex produces the complementary minus-strand RNA. This again functions as template for the positive-sense RNA and subgenomic RNA (Shirako and Strauss 1994). The later replication complex, formed by fully processed nsP1-4 produce the positive-sense genomic and subgenomic RNA (Figure 5).

The alphavirus structural proteins, glycoproteins E1, pE2 (E2 and E3) including capsid and 6K, are translated from the subgenomic 26s mRNA as a polyprotein. The first structural protein, the capsid, is first released (37) into the cytoplasm, where it binds to several other viral components to form viral particles. The remaining part of the unprocessed polyprotein gets translocated into the endoplasmic reticulum (ER) lumen, with most of the protein located on the inside (Figure 5). Here pE2, 6K and E1 is liberated from each other (38) and heterodimers of pE2 and E1 are made in the ER before transportation to the plasma membranes through the Golgi apparatus (39). In the plasma membranes pE2-E1 assemble into trimers and pE2 is cleaved into E2 and a small glycoprotein E3, which carry the signal for the translocation of pE2 into the lumen of ER (40). The 6K proteins form ion channels in cell membranes and increases membrane permeability which facilitates virus budding (41-43). For SAV3 it has been shown to be important for virus assembly or budding as viral proteins were produced, but no viable virus was released from cells transfected with a cDNA plasmid lacking the entire 6K (44).

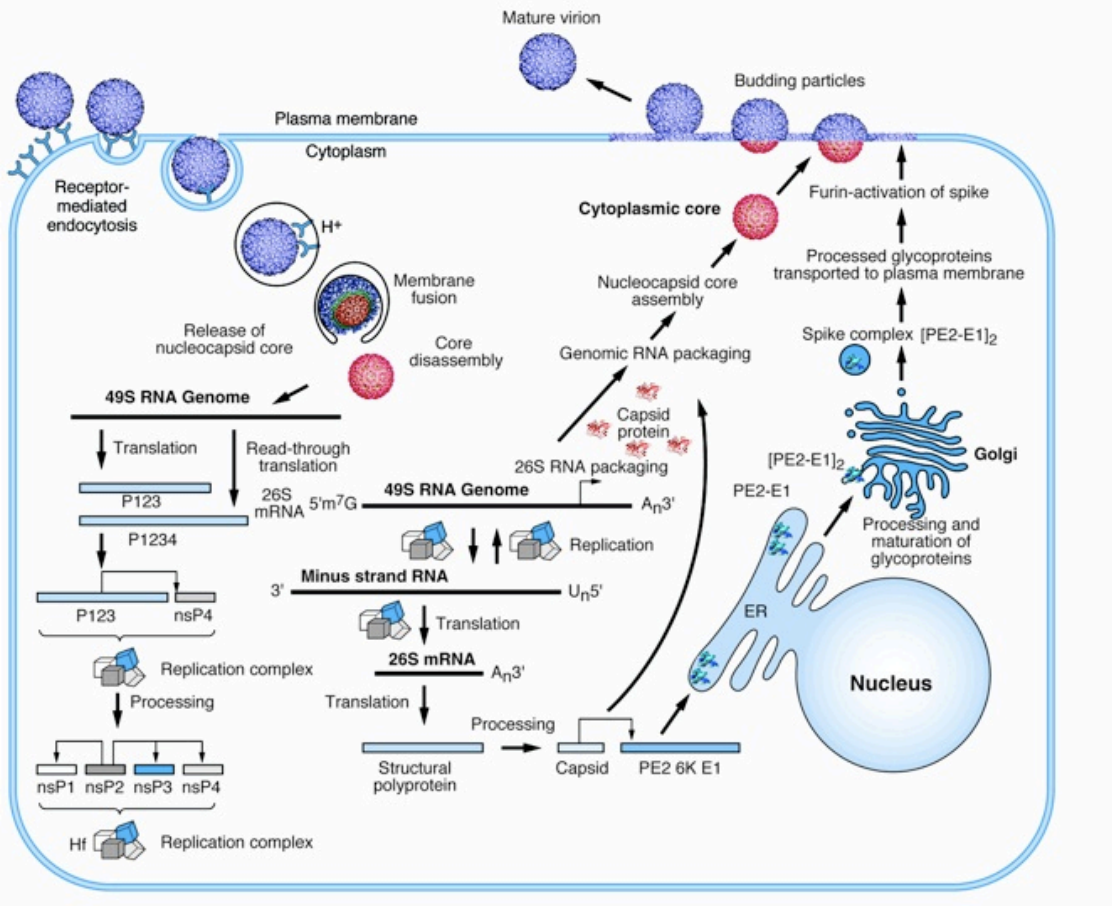

Figure 5. Alphavirus replication cycle. Viral entry through receptor-mediated endocytosis. Low pH in the endosome triggers viral fusion and nucleocapsid is released into the cytoplasm. The nonstructural proteins, nsP1, 2, 3 and 4, along with host factors, form the cellular membrane-bound replication complex that performs the replication of the viral genome and transcription of the RNA. During virus budding the nucleocapsid cores that are assembled in the cytoplasm interact with the envelope glycoproteins at the plasma membrane to form virions. Printed with permission from Dr. R.J Kuhn.

Recombination

Recombination in RNA viruses can occur in both segmented and non-segmented viruses. In segmented viruses the reassortment between RNA segments contributes to antigenic shift and selection of certain phenotypes like for influenza virus. In non-segmented viruses and in single segments of segmented viruses the exchange of genetic information is between RNA molecules.

A recombinant sequence can be conceived in two different ways; either via breaking the parental sequences and joining the resulting fragments or via the copy choice model where the polymerase disassociate or switches to another template, continuing the copying on the new template while holding on to the nascent strand generated initially (Figure 6). The latter has been the most commonly accepted model for RNA recombination (45) after the work of Kirkegaard and Baltimore on homologous recombination in poliovirus (46). Nonreplicative RNA recombination has also been demonstrated experimentally (47, 48) but at a much lower frequency than copy choice. It occurs when RNAs are cleaved at specific points and ligated to form hybrid molecules. Enzyme mechanisms are thought to be involved and RNA secondary structure rather than sequence similarity is expected to be the major factor mediating nonreplicative recombination.

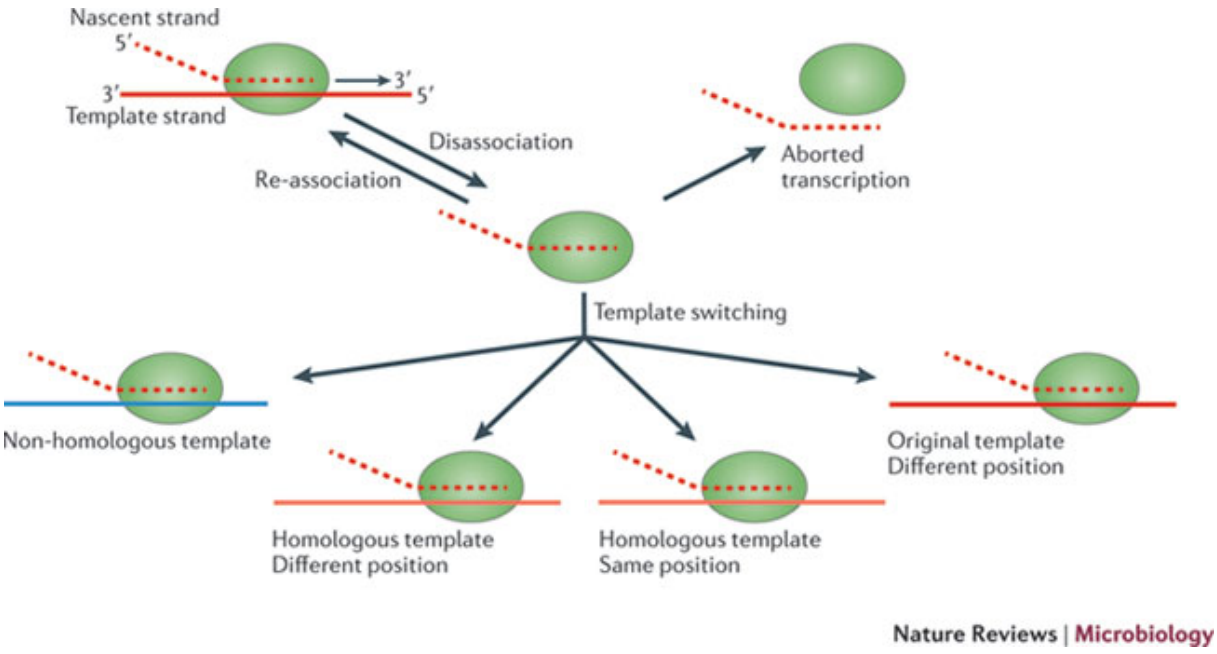

Figure 6. Illustration of the potential outcomes of a template switching. The polymerase might bind to a non-homologous or homologous template at the same or at different position. Reprinted with permission from Nature Reviews Microbiology.

RNA recombination in non-segmented viruses are traditionally classified into:

- Homologous recombination, which involves two similar or closely related RNA molecules with extensive sequence homology. Crossover occur at matched sites so that the recombinant RNAs retain the exact sequence and structural organization as the parental RNA molecules. Homologous recombination are the most likely to generate functional progeny.

- Aberrant/imprecise homologous recombination also involves two RNA molecules with similar sequences, but the crossover occurs at unrelated although usually nearby sites resulting in sequence duplication or deletion.

- Non-homologous recombination occurs on RNA molecules that does not show any sequence homology. This is the most infrequent RNA recombination and the basis of selection of recombination is unclear but might involve secondary structure similarities.

Aberrant or imprecise homologous recombination is particularly common when defective RNAs are involved in recombination (49). Defective interfering (DI) RNAs are highly deleted forms of the infectious genome that are made by most families of RNA viruses. DI RNAs retain replication and packaging signals, are synthesized preferentially over infectious genomes, and are packaged as DI virus particles (DIPs) which can be transmitted to susceptible cells. Their ability to interfere with the replication of infectious virus in cell culture by accumulation, disease attenuation and their potential as antivirals have long been known (50). In general, DI RNAs are created spontaneously and multiply rapidly. They are commonly produced during *in vitro* passages at high numbers of virus particles to the

number of target cells (MOI) and slow down the parent virus´ multiplication. The RNAs are called "defective" because they have lost the capacity to code for all the necessary viral proteins for independent replication.

Recombination in Alphaviruses

Recombination in alphaviruses was first demonstrated *in vitro* for SINV in 1991 by the work of Weiss and Schlesinger (51) using replication competent SIN virus RNAs transcribed from cloned cDNA plasmids, where near wild type SIN genomes was produced from two smaller cDNA plasmids carrying each of the ORFs. Further documentation was reported in the nineties, although only in *in vitro* systems (52, 53). In natural infection with alphavirus the recombination event between EEEV and SIN like viruses, resulting in WEEV, Highlands J virus and Fort Morgan virus (54, 55) is evidence for its natural existence and contribution to viral evolution. DI RNAs or DI particle formation is also known in alphaviruses (56-58), but few have been reported from natural infections ((paper 2), (59). Intrahost variability can generate different kinds of mutations including deletions and insertions, but deletions seems to be more common for alphaviruses.

AIMS OF THE STUDY

The specific aims of the study were:

1. To better understand SAV3 mechanisms of virulence through tissue culture adaptation

2. To provide sequence analysis of full-length viral genomes of SAV3 from natural infections and to explore the divergence between the Norwegian strains

3. To document alphavirus RNA-RNA recombination using an animal model

SUMMARY OF SEPARATE PAPERS

Paper I

The first paper show that cell culture of SAV3-H10 selects for strains inducing earlier CPE *in vitro* with increased viral replication. A Norwegian subtype SAV3 virus isolate (SAV3-H10) was subjected to serial passages in CHSE-214 followed by AGK. Two passages from CHSE and one after transfer to AGK cells were chosen for further investigation, based on variation in passage number and degree and development of cytopathic effect (CPE). After plaque purification several *in vitro* studies were performed. The AGK-transferred isolate was identified with the strongest abilities to reduce cell viability, replicate more and cause more CPE in cell culture when compared with the early and late CHSE-grown isolates. Subsequently, the isolates were tested in an experimental fish challenge, showing higher viral load and higher pathological score for the least cell-cultured isolate. Full-length sequencing of the viral genome of the three isolates revealed divergence in four amino acid positions and the AGK-grown isolate also had a 3 nt deletion in the 3´UTR. In summary, the effect of cell culture adaptation resulting in increased replication and CPE to the cells was reversed *in vivo*, resulting in lower replication levels and lower pathology scores in target organs. It is suggested that the changes in the 3'UTR region are relevant to the adaptation to cell culture conditions of AGK cells. This study outlined a path to identify potential virulence motifs of SAV3.

Paper II

At the time this study was initiated, relatively few full-length genome sequences of SAV3 strains were available. This paper reports full-length genome sequences of nine SAV3 strains from sites farming Atlantic salmon geographically spread along the Norwegian coastline. The virus genomes were sequenced directly from infected heart tissue, to avoid culture selection bias. Sequence analysis confirmed a high level of sequence identity within SAV3 strains, with a mean nucleotide diversity of 0.11 %. Sequence divergence was highest in 6K and E2, while lowest in the capsid protein and the non-structural proteins (nsP4 and nsP2). In addition, this study reported for the first time that numerous defective viral RNA containing genome deletions are generated during natural infection with SAV. Deletions occurred in the isolated RNA from all virus strains and were not distributed randomly throughout the genome but instead tended to aggregate in certain genomic areas. We suggested imprecise homologous recombination as an explanation for generation of the defective viral RNA with deletions. Primary virus isolation was successfully achieved for only two of eight strains, despite extensive attempts using three different cell lines. The presence of these viral RNAs, provides a possible explanation for the difficulties in isolating SAV in cell culture.

Paper III

With the discovery of SAV3 defective RNA in paper 2 and the documentation of *in vitro* recombination of SAV3 RNA by colleagues in 2014, this study was initiated to document SAV3 RNA recombination experimentally *in vivo*. RNA recombination in non-segmented RNA viruses is important for viral evolution and documented for several virus species, but only through *in vitro* studies. Atlantic salmon were injected with a SAV3 6K-gene deleted cDNA plasmid, encoding a non-viable variant of SAV3, together with a helper cDNA plasmid encoding structural proteins and 6K only. In the weeks subsequent to injection, SAV3-specific RNA was detected and recombination of viral RNAs was confirmed. Virus was isolated from plasmid-injected fish, using cell cultures and further shown to infect and cause pathology in salmon. Subsequent sequencing of PCR products confirming recombination, documented imprecise homologous recombination creating RNA deletion variants in fish injected with the cDNA plasmids. Prediction of the RNA secondary structure indicated that deletions are initiated at loops of unpaired nucleotides. Some of the deletions have previously been identified in SAV3 RNA from field infections in salmon, shown in paper 2. This was the first experimental documentation of alphavirus recombination in an animal model.

METHODOLOGY

The experimental methods used in this thesis are all basic and well-known molecular methods. In the first paper primary isolation, propagation and *in vitro* studies of the virus was the main focus. We studied cellular morphology over time after infection by using phase contrast microscopy and a viability assay to indirectly measure viable and metabolically active cells. Viral replication kinetics in cells over time was analyzed by real-time PCR. An *in vivo* study was performed as a final step with evaluation of histopathological changes in heart, pancreas and skeletal muscle after hematoxylin and eosin staining of tissue specimens. Viral load in heart and kidney tissue was measured with real-time PCR.

Further in paper 2, the sampling and analyzing of virus genomes from outbreaks was performed with PCR based techniques, subsequent cloning and sequencing. Standard primary isolation and propagation of the field isolates were also attempted.

Paper 3 started with an *in vivo* study and included both real-time PCR and histopathological evaluation followed by isolation and propagation of the virus in cell culture. Further, basic PCR, cloning and sequencing techniques was used to analyze the material, from both the *in vivo* and cell culture experiments, before a second *in vivo* study was performed including the same methods as the first *in vivo* study.

Primary isolation and propagation of SAV

Primary isolation of SAV from clinically diseased fish is notoriously challenging. Several cell lines have been tested to optimize the isolation including Chinook salmon embryo cells (CHSE-214), Asian Grouper skin cells (AGK) and Chum salmon heart cells (CHH-1). Different conditions e.g. temperature, amount of inoculate and size of wells have been tried to optimize the viral replication (paper 1 and 2). In general, CHH-1 cell line was found to be the best suited. It has been documented that the IFN

response from SAV3 infection is lower in CHH-1 cells compared to CHSE-214 and BF-2 cell lines (44), which might facilitate infection and the initial rounds of virus replication.

In vitro studies

The viability of the cells after infection was studied visually by light microscopy and quantitatively by the MTS assay which measures metabolically active cells over time (paper 1). The results was related to the increase in viral RNA in the cells.

PCR, cloning and sequencing of SAV3 field isolates

The initial goal of the study in paper 2 was to do full-length sequencing of field isolates of SAV3. Since the genome is relatively large (almost 12 kb) amplification of a full-length genome PCR product with subsequent cloning is troublesome. Instead, 6 sequence overlapping PCR fragments were amplified. Attempts to compile sequences directly from the PCR product resulted in poor sequence quality, represented by double/mixed sequence chromatogram peaks. To overcome this, cloning of the PCR products before sequencing was performed. At that time we did not know that deletion mutants would dominate the sequences obtained which possibly explain the difficulties of direct sequencing of PCR products.

From cDNA plasmid to infectious virus

Our group have earlier documented RNA recombination *in vitro* (44), using a SAV3 6K-gene deleted cDNA plasmid, encoding a non-viable variant of SAV3, together with a helper cDNA plasmid encoding structural proteins and 6K only. Since the cDNA plasmids of that study was available, they were used in the experiments constituting the study of paper 3 with an aim to document RNA recombination *in*

vivo. To document that two injected plasmids resulted in an infectious virus, several steps was necessary. Initial real-time PCR analyses of plasmid-injected fish was performed. Procedures were undertaken to verify that the amplification products was not direct amplification from injected plasmids. PCR amplification and sequencing confirmation of a product including sequences unique for both plasmids was necessary to confirm a recombination event. To document an infectious virus, isolation and propagation on CHH-1 cells was performed. Further, to show that the virus also induced pathology similar to wild type SAV3 a second *in vivo* study was conducted.

RESULTS AND GENERAL DISCUSSION

SAV infection and PD outbreaks are known to cause variable levels of mortality, as seen e.g. in a study of Atlantic salmon sites in Norway from 2006-2008, where mortality varied from insignificant and up to more than 25% (60). The infection in general induce similar histological lesions, but virulence between strains and also subtypes is variable (8, 17, 61). Molecular determinants of SAV virulence, which might cause differences in mortality, has not been defined, although two amino acid substitutions in the E2 glycoprotein of SAV2 are associated with virulence in rainbow trout (62). An *in vitro* study has suggested other amino acid substitutions related to virulence (63), e.g. in the E2 gene, but it seems that adaptation to cell culture does not mirror *in vivo* virulence (64).

In paper 1 we document the genetic change on a CHSE adapted virus isolate when transferring it to a new environment, the AGK cell line, and describe amino acid substitutions in E2, E1 and nsP1. It was discussed and suggested that the substitution in E2 (Q133K) could play a role, e.g. in receptor binding efficiency, and be related to the increased virulence in AGK cells. Still, it was shown that the AGK adapted passage strain with this substitution actually had the lowest viral load at day 0 p.i. in both cell lines, pointing towards a low receptor binding efficiency. Although starting with lower viral load, this passage strain reached the highest end point, reflecting that other factors than cell receptor binding plays a role, e.g. genome replication and transcription efficiency. The 3 nt deletion in the 3´UTR of the AGK adapted passage could be related to this. RNA secondary structure predictions of the 3´UTR with and without these 3 nt deleted (Figure 7) indicate that this deletion results in pronounced secondary structure changes. This change might well be a result of adaptation produced by a natural selection of the most fit virus, resulting in a more replication efficient virus. The 3´UTR of alphaviruses and in particular CSE4, a sequence structure that are conserved among alphaviruses, is believed to be important in regulating minus-strand RNA synthesis (19). The 3nt deletion is located in the region of SAV3 3'UTR, that putatively hold a function similar to other alphavirus CSE4. Paper 2 describes amino acid variations possible representing putative virulence motifs between field infection strains, but

identifying virulence motifs among these would require more in-depth studies using reverse genetics and *in vivo* challenge of susceptible fish.

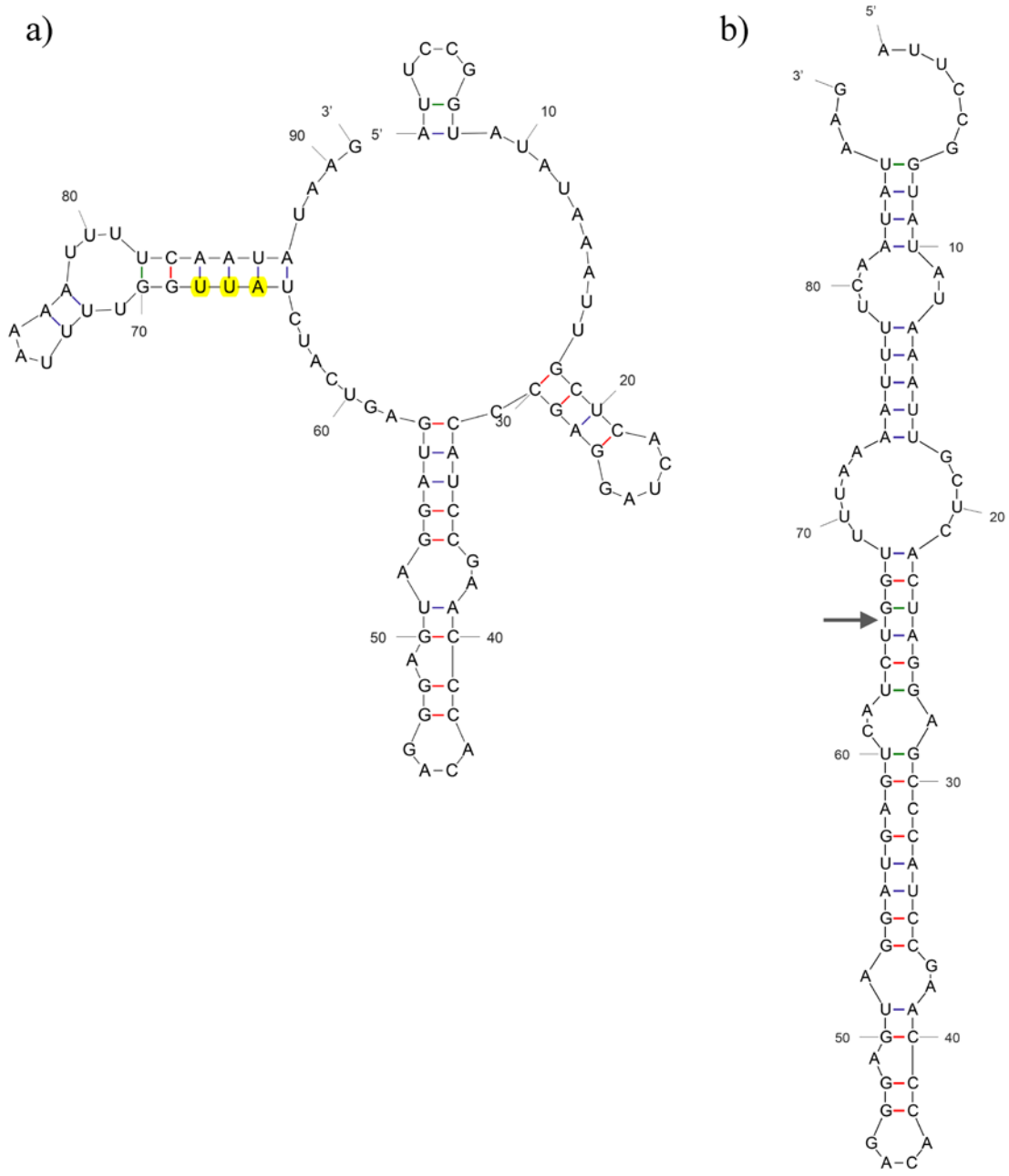

Figure 7. Predicted RNA secondary structure using Mfold of 3´UTR. a) CHSE adapted passage. The 3nt deleted in b) are highlighted in yellow. b) AGK adapted passage with arrow indicating the position for the deleted nts.

Adaptation and attenuation following passage *in vitro* using cell lines, is a well-known phenomenon of viruses, also among alphaviruses, and has been useful in the development of live vaccine strains (65-67). Since primary isolation of SAV from field outbreaks has been challenging, adaptation has been discussed as a necessity for isolation in cell culture (10, 12, 68). In paper 1 we document that SAV3 adaptation to AGK cell culture leads to decreased virulence *in vivo* which illustrates the importance of differentiating between *in vivo* and *in vitro* virulence. The *in vivo* system represents a much more complex biological system than the single cell type environment provided by cell culture systems, and thus fitness criteria differ. Retrospective, it would have been interesting to sequence directly the virus, as performed in paper 2, prior to cell culture passaging on CHSE and AGK cells, and also to explore adaptation in other cell lines. The possibility of reversion could also be tested by transferring the virus back to CHSE after AGK adaptation.

When the study in paper 2 was started, only a few SAV full-length sequences were published and most of these were obtained from cell cultured isolates. In addition, a high number of partial sequence data were available, however mainly focusing on smaller parts of the viral genome. Sequences derived directly from the fish would represent fingerprints of disease-causing motifs with no interference from cell culture adaptation. The sequence data of the full SAV3 genome achieved directly from heart tissue presented in paper 2, provides a detailed description of SAV3 genome diversity including strains geographically found along the whole Norwegian coast and confirms the common understanding of only minor genetic variation within SAV3 subtype (16, 63, 69). In paper 1 the effect of adaptation to cell culture was documented together with the importance of *in vivo* based information, confirming that sequences directly from field infections is an important contribution to a SAV3 genome database.

RNA recombination in alphaviruses has been described in the 90s with Sindbis virus (SINV) as the most studied prototype. Transfection of permissive cells or *in vitro* systems, have been used to confirm recombination events, which was also done for SAV3 by our group in 2014 (44). Based on the *in vitro*

based knowledge of RNA recombination in SAV3 and the indications of recombination *in vivo* from the observation of numerous defective viral RNA containing deletions from field material in paper 2, the study describing recombination of SAV3 RNA *in vivo* in paper 3 was initiated. The same experimental templates for transcription of viral RNA as used by Guo et al (44) was applied, but the host was switched from cell culture to Atlantic salmon where it was injected into the muscle.

For alphaviruses, imprecise recombination resulting in defective interfering (DI) RNAs or DI particles (DIPs) are known (56, 58). The parent virus or a helper virus is required to provide missing genes for replication proteins. The DI-RNAs are "interfering" because they can attenuate the symptoms caused by the helper virus (70). However, some defective RNAs do not interfere with multiplication of their helper viruses. Viral RNA with deletions not defined as DIPs have been documented for VEEV (59) and is also indicated for SAV3 in paper 2. In this thesis there is no documentation as to whether the deleted RNAs are packed into particles or not, and the effect on the replication of parental virus is unknown and not studied. In contrast to DI RNA during serial passage, which accumulate with relation to high viral titers, we have indications that the viral RNA carrying the deletions are diluted rather than multiplying. In paper 2 we document their presence in natural infections in the salmon host, which generally occur at low infection titer (paper 2) and in paper 3 they are produced experimentally *in vivo* after injecting cDNA plasmids encoding a non-viable variant of SAV3, together with a helper cDNA. Several alphavirus deletion mutants have been generated *in vitro*, e.g. mutants with deletions in the 6K protein of viable SINV strains, however such deletions have not been identified in natural populations of SINV. For SAV1 the published genomic sequence represents a deletion mutant that lacks 108 nt in the 6K region (11). The official full-genome sequence of the isolate was later corrected to include the deleted 108nt, but it was suggested that the deletion in the 6K gene was real and also that deletions could possibly be present in other regions of the genome (14), which now is documented in paper 2. *In vitro* studies of both flavivirus and alphavirus DI RNAs are related to persistent infections (58, 71, 72), but not shown by *in vivo* experiments. The nature of the deletion mutants documented in this thesis seems in many ways in contrast to the known nature

of DI RNAs and DIPs. The majority of the deleted RNAs documented in paper 2 and 3, result in a frameshift in the associated ORF and are most likely non-viable. If the deleted RNA is not encapsidated into virus particles, it will not be transferred and infect other hosts, and only be part of an intrahost or even intra cellular variation of the virus. It has been theorized that deletions can be generated as a by-product of replication, with no advantage to the viral population (73)(ref?). This would be a result of the low-fidelity polymerase, and occasionally the deletions would result in beneficial combinations. Future research should include determining whether the deletion mutants simply are defective RNA or if they interfere and/or is encapsidated into virus particles.

It has been hypothesized that RNA-dependent RNA polymerase fidelity is tightly linked with recombination and DI production and that high viral titers result in more DIs and attenuation of the virus (57). We find numerous deletion mutants of SAV during natural infection, and when these wild-type isolates are reisolated in cell culture, the deleted RNAs seem to disappear with passaging of the virus (68) (paper 3). Culturing *in vitro* could be an environment that "purifies" the isolates, with declining concentration of truncated RNAs present for every passage. However, the propensity for deletions to occur in the SAV genome is obviously high and deletion variants reappear after injection of the "purified" virus isolate cell supernatant intramuscularly into fish, as shown in paper 3. In this paper, the prediction of RNA secondary structure clearly shows that deletions are initiated in areas where the structure changes from loops of unpaired bases to basepair helix secondary structures. The pausing of the polymerase at secondary structures might lead to a release of the RNA polymerase from the template strand, but also nonreplicative mechanisms like splicing reactions or cleavage and ligation of RNA might occur. The fact that viral deletion mutants produced under field infections of SAV3 in Atlantic salmon correlates with deletions found experimentally *in vivo* in fish injected with SAV3 cDNA plasmids, states that RNA recombination and the production of defective RNA is a strong and consequent feature contained in the SAV genome.

The history of poliovirus vaccines illustrates the potential consequences using attenuated vaccines for viruses that recombine *in vivo*. The ability of polio virus strains in vaccines to exchange genetic material was first described in 1984 by Kew and Nottay (74) and raised the issue of the stability and safety of the vaccine. Such recombinants were found to appear very frequently (75-77), mostly in patients with vaccine-associated paralytic poliomyelitis (VAPP). In some cases, recombinants between vaccine and wild strains have also been isolated from VAPP cases (77, 78). Recombination events and increased pathogenicity has been revealed on multiple occasions with other viruses (79-81) and recombination or reversion in RNA viruses for which vaccines were in use or in experimental trials, has also been seen (82-84). The confirmation of *in vivo* recombination of viral RNA in Paper 3, illustrates that also for SAV and possibly alphavirus in general, the safety regarding attenuated vaccines and vaccines containing full-length or partially deleted viral genomes should be evaluated against the frequency and potential consequences of recombination events. There is also potential for new pathogenic hybrid strains of SAV if RNA from the vaccine and wild-type virus strains infecting the vaccinated host recombine. Several SAV replicons have been made to express heterologous antigens of other salmonid viruses, like IPNV and ISAV (85-88) with the purpose to be used for vaccination. A potential hybrid SAV carrying antigens of another virus would be the most extreme consequence of SAVs recombination events.

The documentation of experimental RNA recombination *in vivo* is the first ever for alphaviruses and might give a new perspective to the evolutionary history of piscine alphaviruses. The geographical origin of the alphaviruses has been debated and a virus ancestor from the Americas or Africa have both been suggested (30, 54, 89, 90). Others suggest a marine ancestor (30, 91, 92). The SAV subtypes seem to have been separately introduced to the salmon farming industry indicating a common ancestor in the North Sea and a marine ancestor of alphaviruses is thus not unlikely (92). Its theorized that the marine ancestor emerged to terrestrial vertebrate hosts and after subsequent movements both east and west the ancestors of the mosquito borne viruses would be the results (91). Yet another study

suggests that the virus initially infected insects (30). SINV proteins nsP1, nsP2 and nsP4 have been shown to share homology with several RNA plant viruses. Fundamental similarities in replication mechanisms supports an evolutionary relationship, likely descended from a common ancestor (93, 94). The fact that New World alphaviruses can replicate in fish cells at lower temperatures (95) illustrates that the evolutionary history of the genus is complex, but very likely involves several recombinatorial events. If the subtypes of SAV have a common ancestor, recombination probably explains the development of the different subtypes.

An intra-host genetic pool with viral deletion mutants as demonstrated in paper 2 and 3 could provide a genetic plasticity and be beneficial for cross-species transmission ("host-jumping"). Although the wild reservoir of SAV still remain elusive the finding of SAV5 in wild caught fish species (96) reflects the viral host potential, which probably is broader than the vector transferred alphaviruses. In addition, the separate introductions of the six SAV subtypes from a wild reservoir in or around the North Sea supports this. The indications of a marine alphavirus ancestor (91) fits well with the theory of an ancestor with a wide host specter, ability to recombine and evolving to become terrestrial. Interestingly SAV have a stretch of nucleotides in the genome, encoding a unique seven amino acid domain in 6K protein, when compared to EEEV, SINV, SFV, WEEV and Ross River virus (RRV) (44). It is tempting to speculate that this could be a result of an imprecise recombination event in SAV RNA of a marine ancestor resulting in a deletion transferred over time to the other alphaviruses. Between SAV1 and SAV2 a 24nt deletion in the nsP3 of SAV2 is the most notable distinguishing feature between SAV1-3 subtypes (14, 69) and in general insertion/deletions are present between SAV1-3. Since recombination and deletions seems to be a common feature of SAV this will clearly also affect the future evolution of the virus. With today's situation in Norway where two subtypes exists, partially in the same geographical area, it is tempting to speculate in future recombination events, leading to SAV chimeras.

MAIN CONCLUSIONS

This thesis has provided an overall better understanding of SAV3 mechanisms of virulence through tissue culture adaptation. Sequences of full-length viral genomes of SAV3 from natural infections is provided and confirms the low divergence between the Norwegian SAV3 strains on a better basis not affected by cell culture bias. RNA recombination in an alphavirus was documented for the first time in an animal model. More specifically the thesis documents:

- Passage of SAV3 in two different cell lines leads to adaptation. The change of cell line had a higher impact on adaptation than passaging. *In vitro* adaptation induces earlier CPE with increased viral replication. In contrast, the effects is reversed *in vivo* resulting in delayed onset of pathology in target organs in parallel with lower virus replication levels. Variation was found at four amino acid positions, in addition to a 3 nt deletion in 3`UTR.

- Natural infection of SAV3 generates numerous viral RNA copies with internally deleted genomes. Full-genome consensus sequences of SAV3 shows homogeneous genetic fingerprint.

- SAV3 RNA recombination can occur *in vivo* in Atlantic salmon after injection of replication incompetent cDNA plasmids. The recombination produces a full-length virus, which is infectious in cell culture and induces pathology when injected into Atlantic salmon.

- The RNA recombination is imprecise and creates RNA deletion variants that occur related to loops of unpaired nucleotides in the RNA secondary structure. Deletions found in viral RNA from plasmid injected fish correlates with deletions found in SAV3 field strains.

IMPLICATIONS FOR FUTURE RESEARCH

The initial work of this thesis has focused on virulence and adaptation. *In vitro* adaptation of SAV3 was confirmed, but the underlying patterns for understanding the virulence of SAV3 still remain elusive. The determination of single residues or combination of residues defining SAV3 virulence *in vivo* should be addressed through reverse genetics and *in vivo* studies. This thesis show that the 3'UTR might have an influence on virulence and although it also adds knowledge on the variability of viral genome sequences, the 3'UTR was not included in the studies of field strains and should therefore be paid attention in future research on genome sequence motifs related to virulence.

Research and diagnostics use real-time PCR and resulting Ct-values to study the presence and relative variation in the viral load in sampled material related to SAV3 infection. The impact of the presence of the defective RNA on Ct values and the possible bias should be given some attention, as the Ct-values might not represent level of RNA from viable virus. A selection of the samples from individuals with a low Ct value (presumably indicating high viral loads) is often used for primary virus isolation attempts. The observed difficulties isolating virus from tissue might be due to a high concentration of defective RNA relative to the full-length viable RNA genome, which could indicate that presence of deletion mutants is of some relevance to replication and packaging/release of the virus. More studies dedicated to the prevalence, characteristics and impact of these deletions are needed, and the role of defective RNA or possible defective interfering RNA for the persistence of SAV3 infection should be further investigated.

REFERENCES

1. Council NS. [cited 2016. Available from: http://en.seafood.no/News-and-media/News-archive/Press-releases/Norwegian-salmon-and-trout-exports-worth-NOK-50-billion-in-2015.
2. The Norwegian Ministry of Trade IaF. Aquaculture 01.01.2014 [cited 2016. Available from: http://www.fisheries.no/aquaculture/Aquaculture/.
3. Gudding R, Van Muiswinkel WB. A history of fish vaccination: science-based disease prevention in aquaculture. Fish ShellfishImmunol. 2013;35(6):1683-8.
4. Press CM, Lillehaug A. Vaccination in European salmonid aquaculture: a review of practices and prospects. BrVetJ. 1995;151(1):45-69.
5. Munro ALS, Ellis AE, Mcvicar AH, McLay AH, Needham EA. An exocrine pancreas disease of farmed Atlantic salmon in Scotland. Helgolander Meeresuntersuchungen. 1983(37):571-86.
6. Poppe T, Rimstad E, Hyllseth B. Pancreas disease in atlantic salmon (*Salmo salar*) postsmolts infected with infectious pancreatic necrosis virus (IPNV). BullEurAssFish Pathol. 1989;9(4):83-5.
7. Hjortaas MJ, Skjelstad HR, Taksdal T, Olsen AB, Johansen R, Bang-Jensen B, et al. The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, *Salmo salar* L., in Norway. JFish Dis. 2012;36(1):71-4.
8. McLoughlin MF, Graham DA, Norris A, Matthews D, Foyle L, Rowley HM, et al. Virological, serological and histopathological evaluation of fish strain susceptibility to experimental infection with salmonid alphavirus. DisAquatOrgan. 2006;72(2):125-33.
9. Mcvicar AH. Pancreas Disease of farmed Atlantic Salmon, *Salmo Salar,* in Scotland - Epidemiology and early pathology. Aquaculture. 1987;67(1-2):71-8.
10. Nelson RT, McLoughlin MF, Rowley HM, Platten MA, Mccormick JI. Isolation of a Toga-like virus from farmed Atlantic salmon *Salmo Salar* with Pancreas Disease. DisAquatOrgan. 1995;22:25-32.
11. Weston JH, Welsh MD, McLoughlin MF, Todd D. Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, *Salmo salar L*. Virology. 1999;256(2):188-95.
12. Castric J, Baudin-Laurencin F, Brèmont M, Le Ven A, Bèarzotti M. Isolation of the virus responsible for sleeping-disease in experimentally infected rainbow-trout *Oncorhynchus Mykiss*. BullEurAssocFish Pathol. 1997;17:27-30.
13. Villoing S, Bearzotti M, Chilmonczyk S, Castric J, Bremont M. Rainbow trout sleeping disease virus is an atypical alphavirus. JVirol. 2000;74(1):173-83.
14. Weston J, Villoing S, Bremont M, Castric J, Pfeffer M, Jewhurst V, et al. Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. JVirol. 2002;76(12):6155-63.
15. Hodneland K, Bratland A, Christie KE, Endresen C, Nylund A. New subtype of salmonid alphavirus (SAV), *Togaviridae*, from Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* in Norway. DisAquatOrgan. 2005;66(2):113-20.
16. Fringuelli E, Rowley HM, Wilson JC, Hunter R, Rodger H, Graham DA. Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. JFish Dis. 2008;31(11):811-23.
17. Graham DA, Frost P, McLaughlin K, Rowley HM, Gabestad I, Gordon A, et al. A comparative study of marine salmonid alphavirus subtypes 1-6 using an experimental cohabitation challenge model. JFish Dis. 2011;34(4):273-86.
18. Jansen MDB, Jensen B.; Taksdal, T.; Sindre, H.; Lillehaug, A. Pankreassykdom hos laksefisk- en review med fokus på forebyging, kontroll og bekjempelse. The Norwegian Veterinary Institute, 2015.

19. Strauss JH. The alphaviruses: gene expression, replication, and evolution. MicrobiolRev. 1994;58:491-562.
20. Xu C, Guo TC, Mutoloki S, Haugland O, Evensen O. Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon. VetRes. 2012;78(1):1-43.
21. La LM, Gardner J, Warrilow D, Darnell GA, McMahon CR, Field I, et al. Arbovirus of marine mammals: a new alphavirus isolated from the elephant seal louse, *Lepidophthirus macrorhini*. JVirol. 2001;75(9):4103-9.
22. Petterson E, Sandberg M, Santi N. Salmonid alphavirus associated with Lepeophtheirus salmonis (Copepoda: Caligidae) from Atlantic salmon, *Salmo salar L*. JFish Dis. 2009;32(5):477-9.
23. Rodger H, Mitchell S. Epidemiological observations of pancreas disease of farmed Atlantic salmon, Salmo salar L., in Ireland. JFishDis. 2007;30(3):157-67.
24. Levinson RS, Strauss JH, Strauss EG. Complete sequence of the genomic RNA of O'nyong-nyong virus and its use in the construction of alphavirus phylogenetic trees. Virology. 1990;175(1):110-23.
25. Suhrbier A, Jaffar-Bandjee MC, Gasque P. Arthritogenic alphaviruses--an overview. NatRevRheumatol. 2012;8(7):420-9.
26. Nolen-Walston R, Bedenice D, Rodriguez C, Rushton S, Bright A, Fecteau ME, et al. Eastern equine encephalitis in 9 South American camelids. JVetInternMed. 2007;21(4):846-52.
27. Quiroz E, Aguilar PV, Cisneros J, Tesh RB, Weaver SC. Venezuelan equine encephalitis in Panama: fatal endemic disease and genetic diversity of etiologic viral strains. PLoSNeglTropDis. 2009;3(6):e472.
28. Roy CJ, Reed DS, Wilhelmsen CL, Hartings J, Norris S, Steele KE. Pathogenesis of aerosolized Eastern Equine Encephalitis virus infection in guinea pigs. Virology journal. 2009;6:170.
29. McLoughlin MF, Graham DA. Alphavirus infections in salmonids-a review. JFishDis. 2007;30(9):511-31.
30. Powers AM, Brault AC, Shirako Y, Strauss EG, Kang W, Strauss JH, et al. Evolutionary relationships and systematics of the alphaviruses. Journal of virology. 2001;75(21):10118-31.
31. Kuhn RJ. Togaviridae: The Viruses and Their Replication. In: P.M. KDMH, editor. Fields`s Virology. 5 ed2007. p. 1001-22.
32. Ou JH, Strauss EG, Strauss JH. The 5'-terminal sequences of the genomic RNAs of several alphaviruses. JMolBiol. 1983;168(1):1-15.
33. Ou JH, Rice CM, Dalgarno L, Strauss EG, Strauss JH. Sequence studies of several alphavirus genomic RNAs in the region containing the start of the subgenomic RNA. ProcNatlAcadSciUSA. 1982;79(17):5235-9.
34. Ou JH, Trent DW, Strauss JH. The 3'-non-coding regions of alphavirus RNAs contain repeating sequences. JMolBiol. 1982;156(4):719-30.
35. Rossmann.M.G. LLJJXYKRJ. Structural changes of envelope proteins during alphavirus fusion. Nature. 2010;468(7324):705-8.
36. Singh I, Helenius A. Role of ribosomes in Semliki Forest virus nucleocapsid uncoating. JVirol. 1992;66(12):7049-58.
37. Skoging U, Liljestrom P. Role of the C-terminal tryptophan residue for the structure-function of the alphavirus capsid protein. JMolBiol. 1998;279(4):865-72.
38. Garoff H, Huylebroeck D, Robinson A, Tillman U, Liljestrom P. The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. JCellBiol. 1990;111(3):867-76.
39. Soonsawad P, Xing L, Milla E, Espinoza JM, Kawano M, Marko M, et al. Structural evidence of glycoprotein assembly in cellular membrane compartments prior to Alphavirus budding. JVirol. 2010;84(21):11145-51.

40. Lobigs M, Zhao HX, Garoff H. Function of Semliki Forest virus E3 peptide in virus assembly: replacement of E3 with an artificial signal peptide abolishes spike heterodimerization and surface expression of E1. JVirol. 1990;64(9):4346-55.
41. Loewy A, Smyth J, von Bonsdorff CH, Liljestrom P, Schlesinger MJ. The 6-kilodalton membrane protein of Semliki Forest virus is involved in the budding process. JVirol. 1995;69(1):469-75.
42. Melton JV, Ewart GD, Weir RC, Board PG, Lee E, Gage PW. Alphavirus 6K proteins form ion channels. JBiolChem. 2002;277(49):46923-31.
43. Sanz MA, Perez L, Carrasco L. Semliki Forest virus 6K protein modifies membrane permeability after inducible expression in Escherichia coli cells. JBiolChem. 1994;269(16):12106-10.
44. Guo TC, Johansson DX, Haugland O, Liljestrom P, Evensen O. A 6K-deletion variant of Salmonid alphavirus is non-viable but can be rescued through RNA recombination. PLoSOne. 2014;9(7):e100184.
45. Chetverin AB. The puzzle of RNA recombination. FEBS letters. 1999;460(1):1-5.
46. Kirkegaard K, Baltimore D. The mechanism of RNA recombination in poliovirus. Cell. 1986;47(3):433-43.
47. Gallei A, Pankraz A, Thiel HJ, Becher P. RNA recombination in vivo in the absence of viral replication. JVirol. 2004;78(12):6271-81.
48. Gmyl AP, Belousov EV, Maslova SV, Khitrina EV, Chetverin AB, Agol VI. Nonreplicative RNA recombination in poliovirus. JVirol. 1999;73(11):8958-65.
49. Lai MMC. RNA Recombination in Animal and Plant Viruses. MicrobiolRev. 1992;56(1):61-79.
50. Dimmock NJ, Easton AJ. Defective interfering influenza virus RNAs: time to reevaluate their clinical potential as broad-spectrum antivirals? JVirol. 2014;88(10):5217-27.
51. Weiss BG, Schlesinger S. Recombination between Sindbis virus RNAs. JVirol. 1991;65(8):4017-25.
52. Hill KR, Hajjou M, Hu JY, Raju R. RNA-RNA recombination in Sindbis virus: roles of the 3' conserved motif, poly(A) tail, and nonviral sequences of template RNAs in polymerase recognition and template switching. JVirol. 1997;71(4):2693-704.
53. Raju R, Subramaniam SV, Hajjou M. Genesis of Sindbis virus by in vivo recombination of nonreplicative RNA precursors. JVirol. 1995;69(12):7391-401.
54. Allison AB, Stallknecht DE, Holmes EC. Evolutionary genetics and vector adaptation of recombinant viruses of the western equine encephalitis antigenic complex provides new insights into alphavirus diversity and host switching. Virology. 2015;474:154-62.
55. Hahn CS, Lustig S, Strauss EG, Strauss JH. Western Equine Encephalitis-Virus is a recombinant virus. ProcNatlAcadSciUSA. 1988;85(16):5997-6001.
56. Monroe SS, Schlesinger S. Common and distinct regions of defective-interfering RNAs of Sindbis virus. JVirol. 1984;49(3):865-72.
57. Poirier EZ, Mounce BC, Rozen-Gagnon K, Hooikaas PJ, Stapleford KA, Moratorio G, et al. Low-fidelity polymerases of alphaviruses recombine at higher rates to overproduce defective interfering particles. JVirol. 2015.
58. Weiss B, Levis R, Schlesinger S. Evolution of virus and defective-interfering RNAs in BHK cells persistently infected with Sindbis virus. JVirol. 1983;48(3):676-84.
59. Forrester NL, Guerbois M, Adams AP, Liang X, Weaver SC. Analysis of intrahost variation in Venezuelan equine encephalitis virus reveals repeated deletions in the 6-kilodalton protein gene. JVirol. 2011;85(17):8709-17.
60. Jansen MD, Taksdal T, Wasmuth MA, Gjerset B, Brun E, Olsen AB, et al. Salmonid alphavirus (SAV) and pancreas disease (PD) in Atlantic salmon, *Salmo salar L.*, in freshwater and seawater sites in Norway from 2006 to 2008. JFishDis. 2010;33(5):391-402.
61. Taksdal T, Bang JB, Bockerman I, McLoughlin MF, Hjortaas MJ, Ramstad A, et al. Mortality and weight loss of Atlantic salmon, *Salmon salar* L., experimentally infected with salmonid alphavirus subtype 2 and subtype 3 isolates from Norway. JFish Dis. 2014.

62. Merour E, Lamoureux A, Bernard J, Biacchesi S, Bremont M. A fully attenuated recombinant salmonid alphavirus becomes pathogenic through a single amino acid change in the E2 glycoprotein. JVirol. 2013;87(10):6027-30.
63. Karlsen M, Hodneland K, Endresen C, Nylund A. Genetic stability within the Norwegian subtype of salmonid alphavirus (family *Togaviridae*). ArchVirol. 2006;151(5):861-74.
64. Karlsen M, Andersen L, Blindheim SH, Rimstad E, Nylund A. A naturally occurring substitution in the E2 protein of Salmonid alphavirus subtype 3 changes viral fitness. VirusRes. 2014;196C:79-86.
65. Gardner CL, Hritz J, Sun C, Vanlandingham DL, Song TY, Ghedin E, et al. Deliberate attenuation of chikungunya virus by adaptation to heparan sulfate-dependent infectivity: a model for rational arboviral vaccine design. PLoSNeglTropDis. 2014;8(2):e2719.
66. Mckinney RW, Berge TO, Sawyer WD, Tigertt WD, Crozier D. Use of an attenuated strain of venezuelan equine encephalomyelitis virus for immunization in man. AmJTropMedHyg. 1963;12:597-603.
67. Roukens AH, Visser LG. Yellow fever vaccine: past, present and future. ExpertOpinBiolTher. 2008;8(11):1787-95.
68. Petterson E, Stormoen M, Evensen O, Mikalsen AB, Haugland O. Natural infection of Atlantic salmon (*Salmo salar* L.) with salmonid alphavirus 3 generates numerous viral deletion mutants. JGenVirol. 2013;94(9):1945-54.
69. Weston JH, Graham DA, Branson E, Rowley HM, Walker IW, Jewhurst VA, et al. Nucleotide sequence variation in salmonid alphaviruses from outbreaks of salmon pancreas disease and sleeping disease. DisAquatOrgan. 2005;66(2):105-11.
70. Pathak KB, Nagy PD. Defective Interfering RNAs: Foes of Viruses and Friends of Virologists. Viruses. 2009;1(3):895-919.
71. Poidinger M, Coelen RJ, Mackenzie JS. Persistent infection of Vero cells by the flavivirus Murray Valley encephalitis virus. JGenVirol. 1991;72 (Pt 3):573-8.
72. Tsai KN, Tsang SF, Huang CH, Chang RY. Defective interfering RNAs of Japanese encephalitis virus found in mosquito cells and correlation with persistent infection. VirusRes. 2007;124(1-2):139-50.
73. Simon-Loriere E, Holmes EC. Why do RNA viruses recombine? NatRevMicrobiol. 2011;9(8):617-26.
74. Kew OM, Nottay BK. Molecular epidemiology of polioviruses. RevInfectDis. 1984;6 Suppl 2:S499-504.
75. Driesel G, Diedrich S, Kunkel U, Schreier E. Vaccine-associated cases of poliomyelitis over a 30 year period in East Germany. EurJEpidemiol. 1995;11(6):647-54.
76. Furione M, Guillot S, Otelea D, Balanant J, Candrea A, Crainic R. Polioviruses with natural recombinant genomes isolated from vaccine-associated paralytic poliomyelitis. Virology. 1993;196(1):199-208.
77. Cuervo NS, Guillot S, Romanenkova N, Combiescu M, Aubert-Combiescu A, Seghier M, et al. Genomic Features of Intertypic Recombinant Sabin Poliovirus Strains Excreted by Primary Vaccinees. JVirol. 2001;75(13):5740-51.
78. Friedrich F, Da-Silva EF, Schatzmayr HG. Type 2 poliovirus recombinants isolated from vaccine-associated cases and from healthy contacts in Brazil. ActaVirol. 1996;40(1):27-33.
79. Herrewegh AA, Smeenk I, Horzinek MC, Rottier PJ, de Groot RJ. Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. JVirol. 1998;72(5):4508-14.
80. Lopes AM, Dalton KP, Magalhaes MJ, Parra F, Esteves PJ, Holmes EC, et al. Full genomic analysis of new variant rabbit hemorrhagic disease virus revealed multiple recombination events. JGenVirol. 2015;96(Pt 6):1309-19.
81. Vennema H, Poland A, Foley J, Pedersen NC. Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. Virology. 1998;243(1):150-7.

82. Suzuki Y, Gojobori T, Nakagomi O. Intragenic recombinations in rotaviruses. FEBS letters. 1998;427(2):183-7.
83. Worobey M, Rambaut A, Holmes EC. Widespread intra-serotype recombination in natural populations of dengue virus. ProcNatlAcadSciUSA. 1999;96(13):7352-7.
84. Gomez-Casado E, Estepa A, Coll JM. A comparative review on European-farmed finfish RNA viruses and their vaccines. Vaccine. 2011;29(15):2657-71.
85. Wolf A, Hodneland K, Frost P, Hoeijmakers M, Rimstad E. Salmonid alphavirus-based replicon vaccine against infectious salmon anemia (ISA): impact of immunization route and interactions of the replicon vector. FishShellfishImmunol. 2014;36(2):383-92.
86. Wolf A, Hodneland K, Frost P, Braaen S, Rimstad E. A hemagglutinin-esterase-expressing salmonid alphavirus replicon protects Atlantic salmon (Salmo salar) against infectious salmon anemia (ISA). Vaccine. 2013;31(4):661-9.
87. Guo TC, Johansson DX, Liljestrom P, Evensen O, Haugland O. Modification of a salmonid alphavirus replicon vector for enhanced expression of heterologous antigens. JGenVirol. 2015;96(Pt 3):565-70.
88. Abdullah A, Olsen CM, Hodneland K, Rimstad E. A polyprotein-expressing salmonid alphavirus replicon induces modest protection in atlantic salmon (Salmo salar) against infectious pancreatic necrosis. Viruses. 2015;7(1):252-67.
89. Gould EA, Coutard B, Malet H, Morin B, Jamal S, Weaver S, et al. Understanding the alphaviruses: recent research on important emerging pathogens and progress towards their control. AntiviralRes. 2010;87(2):111-24.
90. Weaver SC, Kang W, Shirako Y, Rumenapf T, Strauss EG, Strauss JH. Recombinational history and molecular evolution of western equine encephalomyelitis complex alphaviruses. JVirol. 1997;71(1):613-23.
91. Forrester NL, Palacios G, Tesh RB, Savji N, Guzman H, Sherman M, et al. Genome-scale phylogeny of the alphavirus genus suggests a marine origin. JVirol. 2012;86(5):2729-38.
92. Karlsen M, Gjerset B, Hansen T, Rambaut A. Multiple introductions of salmonid alphavirus from a wild reservoir have caused independent and self-sustainable epizootics in aquaculture. JGenVirol. 2014;95(Pt 1):52-9.
93. Ahlquist P, Strauss EG, Rice CM, Strauss JH, Haseloff J, Zimmern D. Sindbis virus proteins nsP1 and nsP2 contain homology to nonstructural proteins from several RNA plant viruses. JVirol. 1985;53(2):536-42.
94. Haseloff J, Goelet P, Zimmern D, Ahlquist P, Dasgupta R, Kaesberg P. Striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organization. ProcNatlAcadSciUSA. 1984;81(14):4358-62.
95. Wolf.K, Mann.J.A. Poikilotherm vertebrate cell lines and viruses: a current listing for fishes. In Vitro. 1979(16):168-79.
96. Snow M, Black J, Matejusova I, McIntosh R, Baretto E, Wallace IS, et al. Detection of salmonid alphavirus RNA in wild marine fish: implications for the origins of salmon pancreas disease in aquaculture. DisAquatOrgan. 2010;91(3):177-88.

SCIENTIFIC PAPERS

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In vitro adaptation of SAV3 in cell culture correlates with reduced *in vivo* replication capacity and virulence to Atlantic salmon (*Salmo salar* L.) parr

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Salmonid alphavirus (SAV) is the causative agent of pancreas disease affecting Atlantic salmon and rainbow trout and causes a major burden to the aquaculture industry. This study describes a Norwegian subtype SAV3 virus isolate (SAV3-H10) subjected to serial passages in Chinook salmon embryo cells (CHSE-214) followed by Asian Grouper skin cells (AGK). Two passages from CHSE and one after transfer to AGK cells were chosen for further investigation, based on variation in degree and development of cytopathic effect (CPE). After plaque purification, several *in vitro* studies were performed. Cell viability after infection, viral replication and ability to cause morphological changes in CHSE and AGK cells was studied for the three isolates. The AGK-transferred isolate was identified with the strongest abilities to reduce cell viability, replicate more and cause more CPE in cell culture when compared with the early and late CHSE-grown isolates. Subsequently, the isolates were tested in an experimental fish challenge, showing higher viral load and higher pathological score for the least cell-cultured isolate. Full-length sequencing of the viral genome of the three isolates revealed divergence in four amino acid positions and the AGK-grown isolate also had a 3 nt deletion in the 3′UTR. In conclusion, we show that cell culture of SAV3-H10 selects for strains inducing earlier CPE *in vitro* with increased viral replication. *In vivo*, the effect is reversed, with lower replication levels and lower pathology scores in target organs. This study outlines a path to identify potential virulence motifs of SAV3.

INTRODUCTION

Salmon pancreas disease virus (SPDV), also referred to as Salmonid alphavirus (SAV), is the causative agent of pancreas disease (PD) affecting Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) (McLoughlin & Graham, 2007). Pancreas disease is responsible for a large economic loss in many countries as it results in growth retardation and reduced fillet quality in diseased fish. Tissue lesions include degeneration and necrosis of cardiomyocytes, pancreatic acinar cell loss and subsequent skeletal muscle degeneration (McLoughlin *et al.*, 2006; Mcvicar, 1987). Virus has been detected in a wide range of tissues (Andersen *et al.*, 2007), and it has been suggested that pancreas, heart, kidney and spleen are infected at about the same time (Xu *et al.*, 2012). However, the virus replicates with highest viral load in the pancreas and heart (Andersen *et al.*, 2007; Xu *et al.*, 2012), and the pancreas is suggested as the preferred site of replication (McLoughlin & Graham, 2007; McLoughlin *et al.*, 1996; Xu *et al.*, 2012). SAV is currently divided into six different subtypes based on partial sequences of E2 and nsP3 genes (Fringuelli *et al.*, 2008). SAV3 is the subtype found most frequently in Norway, in addition an SAV2-related strain is prevalent as a cause of PD regionally in mid-Norway (Hjortaas *et al.*, 2013).

SAV is a positive-stranded RNA virus of the family *Togaviridae*, genus *Alphavirus*, which consists of an icosahedral nucleocapsid surrounded by an envelope. The virus genome containing two ORFs encodes four non-structural (nsP1, nsP2, nsP3 and nsP4) and five structural proteins (capsid, E3, E2, 6k and E1). E2 together with E1 form heterodimer spikes on the virion surface where E2 is located on the distal portion. Attachment to cells is primarily a function of E2 glycoprotein (Byrnes & Griffin, 1998; Ludwig *et al.*, 1996).

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Primary isolation of SAV3 from field outbreaks has been challenging (McLoughlin & Graham, 2007; Petterson *et al.*, 2013) and adaptation is discussed as a necessity for isolation in cell culture (Castric *et al.*, 1997; Nelson *et al.*, 1995; Petterson *et al.*, 2013). In a study following SAV3 through 20 passages in Chinook salmon embryonic cells (CHSE-214), cytopathic effect (CPE) did not occur until the 13th passage (Karlsen *et al.*, 2006). One of four mutations found at the 20th passage, a serine to proline substitution at E2 position 206, was confirmed to occur coincidently with the CPE in the 13th passage. The substitution was also found in field isolates (Karlsen *et al.*, 2006). A later study using reverse genetics showed that substitution from proline back to serine in this position gave reduced fitness *in vitro*, without notable effects *in vivo* (Karlsen *et al.*, 2015). Recently, it has been demonstrated that two amino acid substitutions in the E2 glycoprotein are associated with a virulent phenotype of SAV2 in rainbow trout (Mérour *et al.*, 2013), but details on molecular determinants of virulence of SAV3 in the fish host and importance of cell culture adaptation still remain elusive.

This study was performed to better understand SAV3 mechanisms of virulence through tissue culture adaptation. CHSE-214 has been utilized as a standard cell line in many SAV studies (Hodneland *et al.*, 2005; Welsh *et al.*, 2000; Weston *et al.*, 2002). Recently, one cell line derived from Asian grouper, named AGK, was investigated for its superiority in infectious pancreas necrosis virus propagation over the use of CHSE-214 cells, due to the cells' fast-growing feature and delayed protein shutdown during the course of infection (Chen *et al.*, 2014). To evaluate its effect on passage of SAV, the AGK cell line was also included in the present study. After serial passage of a SAV3 isolate in CHSE-214 and subsequently in AGK, harvested virus was plaque-purified and the entire genome of the virus from two isolates originating from CHSE-214 only and one after transfer to AGK was sequenced. The plaque-purified isolates of each passage, designated $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$, were examined for *in vitro* characteristics such as ability to induce morphological changes in cell cultures, cell viability post-infection and viral load in infected CHSE-214 and AGK cells. Further, the earliest CHSE- and the AGK-grown isolates were tested experimentally by *in vivo* challenge in salmon. We found that passage in AGK cells results in *in vitro* adaptation with subsequent changes in *in vivo* phenotypes including reduction of virus replication levels and induced pathology in target organs of Atlantic salmon parr.

RESULTS

CPE develops earlier in cells infected with AGK-grown SAV isolate

By starting with heart tissue homogenate supernatant, strain SAV3-H10 was propagated by serial passages until the 11th passage in a CHSE cell line (Fig. 1). Weak signs of CPE (presence of vacuoles) were observed from passage one. From passage five, the CPE developed faster and with increased cell lysis. The 9th passage was then inoculated on AGK cells (originating from a different fish species) and passaged an additional five times (p10 to p14; Fig. 1). CPE was evident from the first passage in AGK cells. Supernatants from an early passage on CHSE ($H10^{P3}$), a late passage on CHSE ($H10^{P11}$) and an AGK-transferred passage ($H10^{P14}$) were subjected to plaque purification and

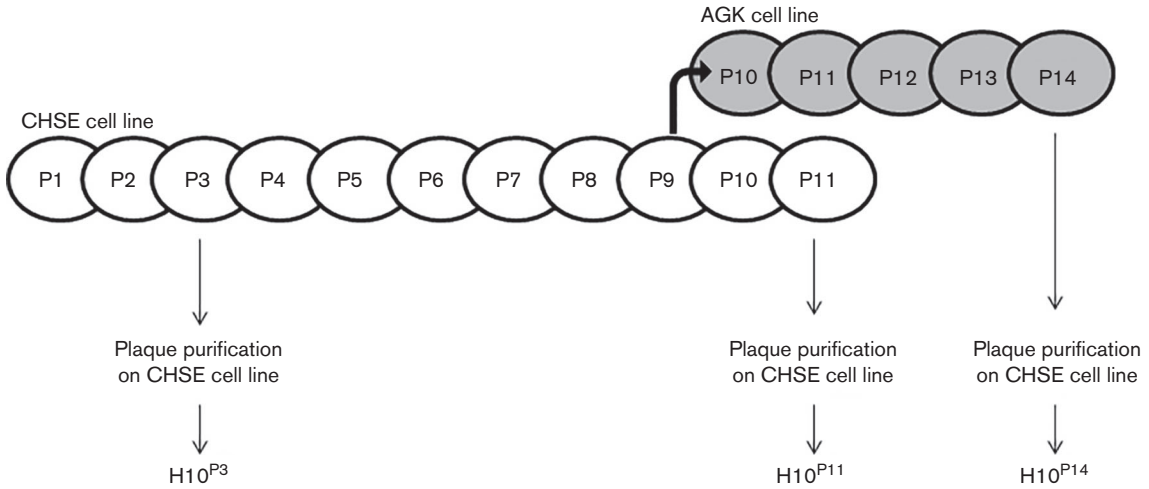

Fig. 1. Overview of SAV3-H10 cell culture passage. SAV3-H10 was subjected to serial passaging in a CHSE cell line combined with an AGK cell line. The 3rd ($H10^{P3}$) and 11th ($H10^{P11}$) passage on CHSE and the passage resulting from nine passages on CHSE followed by five passages on AGK ($H10^{P14}$) were plaque-purified on CHSE, including three passages after plaque selection.

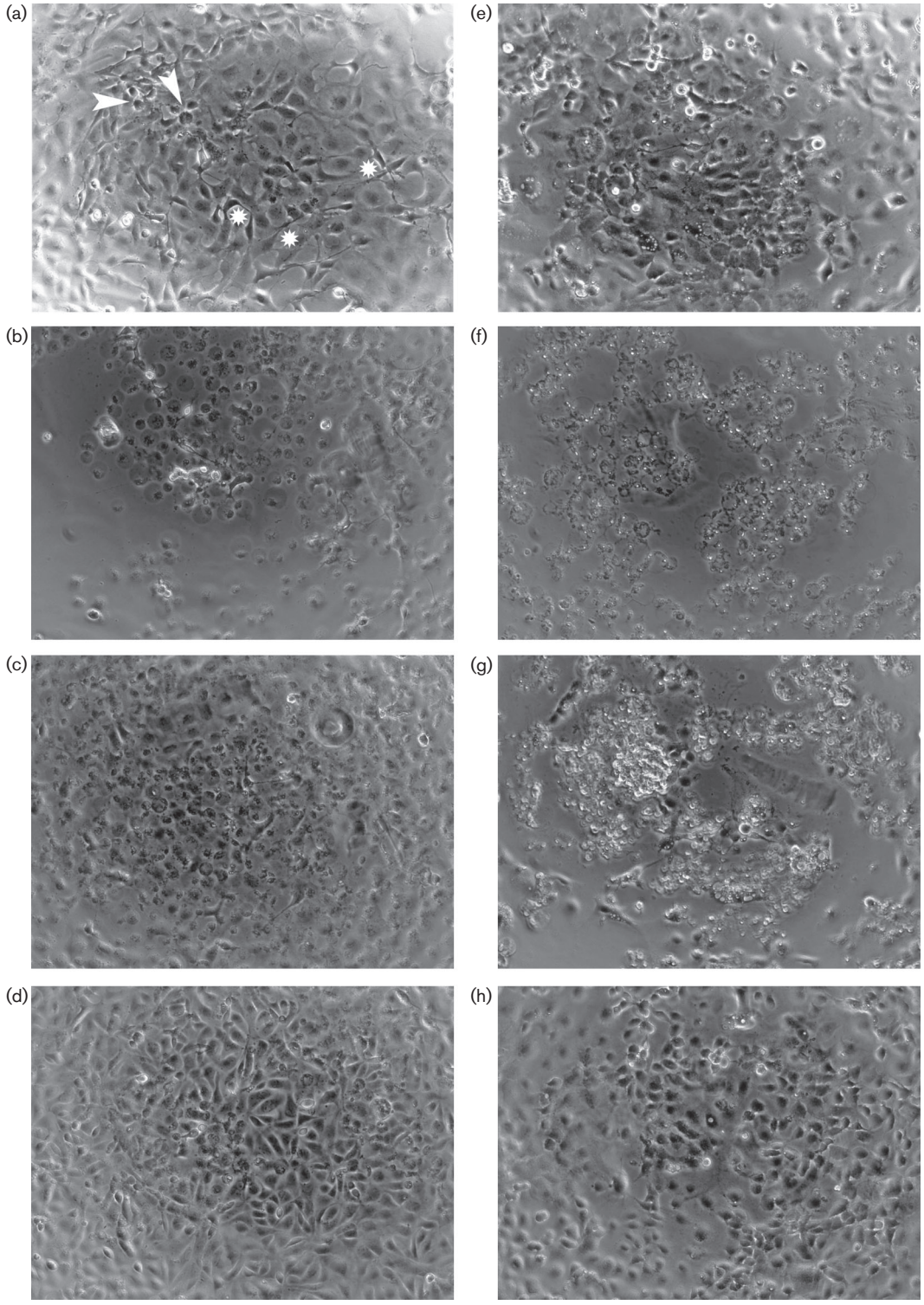

Fig. 2. Examples of cell cultures at days 4 and 14 p.i. with SAV3 $H10^{P14}$ and $H10^{P3}$ at m.o.i. 10. (a) CHSE cells infected with $H10^{P14}$ at 4 days p.i. Shrinkage (*) and swelling (arrowhead) of single cells is indicated. (b) CHSE cells infected with $H10^{P14}$ at 14 days p.i. (c) CHSE cells infected with $H10^{P3}$ at 14 days p.i. (d) CHSE cells non-infected at 14 days p.i. (e) AGK cells infected with $H10^{P14}$ at 4 days p.i. (f) AGK cells infected with $H10^{P14}$ at 14 days p.i. (g) AGK cells infected with $H10^{P3}$ at 14 days p.i. (h) AGK cells non-infected at 14 days p.i.

subsequent characterization. CPE induced by $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$ isolates in CHSE and AGK cells was assessed morphologically by phase-contrast microscopy from 0 h to 14 days post-infection (p.i.). $H10^{P14}$ caused the earliest and most severe CPE of the three strains (Fig. 2), the first signs appearing by day 4 p.i. in both cell lines. In contrast, CPE developed more slowly and was less severe using $H10^{P3}$. CPE developed from $H10^{P11}$ did not differ greatly from that developed from $H10^{P3}$. The pattern of changes was the same for all strains in each cell line, the only difference being the time the CPE occurred. CHSE showed CPE as occasional presence of vacuoles in the first days p.i., followed by shrinkage and then swelling/detaching of single cells, developing into lysis and with a high number of floating, dead cells (Fig. 2a, b). The majority of cells infected with $H10^{P14}$ were lysed by 7 days p.i. with a few cells surviving in all wells at 14 days p.i. (Fig. 2b). For $H10^{P3}$ and $H10^{P11}$, cell-lysis was less prominent towards the end of incubation and shrinkage and/or swelling of single cells together with floating dead cells were the most dominant signs of CPE in CHSE cells (Fig. 2c). AGK cells showed vacuoles for all virus strains at 4 days p.i., although $H10^{P14}$ caused an increased concentration of vacuoles per cell (Fig. 2e). Vacuolization was a characteristic feature in this cell line. Cell lysis and floating cells were dominant at 5–6 days p.i. for $H10^{P14}$ and increased with time p.i. resulting in almost complete lysis (Fig. 2f), while $H10^{P3}$ and $H10^{P11}$ had more viable cells at 14 days p.i. (Fig. 2g).

In vitro cell viability declines earlier in cells infected with AGK-grown SAV isolate

To quantify and substantiate the morphological observations, a viability study was conducted for all strains using m.o.i. 10. $H10^{P14}$ resulted in significant loss of cell viability compared with control cells by 2 and 4 days p.i. in CHSE and AGK, respectively (Fig. 3). $H10^{P3}$ resulted in a loss of viability compared with controls by 6 days p.i. (also significantly different from $H10^{P14}$) in CHSE cells. In AGK cells there was a significant difference between the two strains ($H10^{P14}$ and $H10^{P3}$) and also compared with the control group from day 4 and throughout the study period (Fig. 3b). Differences between the passage isolates appeared as early as day 2 in CHSE (Fig. 3a) and at day 4 in AGK (Fig. 3b). Viability of AGK cells declined from 4 to 8 days p.i. for all strains although this was more pronounced for $H10^{P14}$ (Fig. 3b). Infection using $H10^{P11}$ resulted in an intermediate (or similar to $H10^{P3}$) effect on cell viability compared with $H10^{P3}$ and $H10^{P14}$ in both cell lines (Fig. 3a, b).

AGK-grown SAV showed higher ability for replication in cell culture

The next step was to measure viral replication by real-time PCR. AGK cells were infected with all strains at m.o.i. 10, and CHSE cells with $H10^{P11}$ and $H10^{P14}$ at m.o.i. 10. The

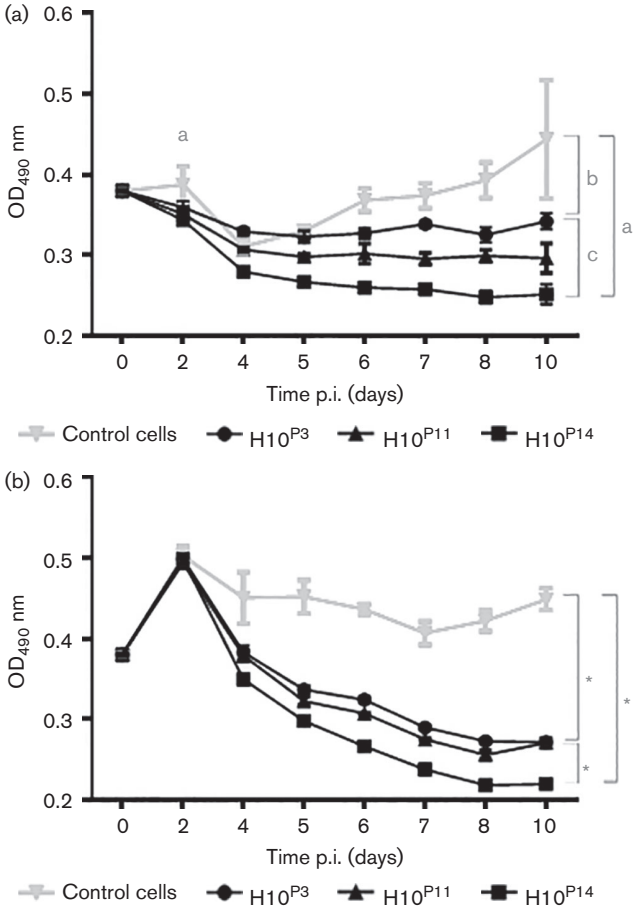

Fig. 3. Cell proliferation in CHSE and AGK cell lines after infection with SAV3 $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$ at m.o.i. 10 measured at OD_{490} as detectable formazan product representing metabolically active cells. Plots show SEM for quadruplicates at each time point. (a) CHSE cell line. a, Significant difference at 2 ($P<0.01$) and from 5 days p.i. ($P<0.001$); b, significant difference from 6 days p.i. (6–7 days p.i., $P<0.05$ and 8–10 days p.i., $P<0.001$); c, significant difference from 4 days p.i. ($P<0.001$). (b) AGK cell line. *Significant difference from 4 days p.i. ($P<0.001$).

results in CHSE indicated that strain $H10^{P14}$ has a higher replication in CHSE compared with $H10^{P11}$ as the levels of viral load increased significantly up to day 4 for $H10^{P14}$, while for $H10^{P11}$ there was no significant increase after day 2. Although, the data indicate that $H10^{P14}$ reached the highest level of viral RNA, the differences from $H10^{P11}$ when compared per sampling time were only statistically significant at day 8 (Fig. 4a). All strains showed significant increase (16-fold, 69-fold and 152-fold for $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$, respectively) in viral load in AGK cells from day 0 to the first sampling at day 2 or 4 (Fig. 4b), and interestingly, viability was not reduced over the first two days (Fig. 3b). Comparing $H10^{P14}$ to $H10^{P3}$ and $H10^{P11}$, there was a significantly higher viral load at days 6, 8 and 10

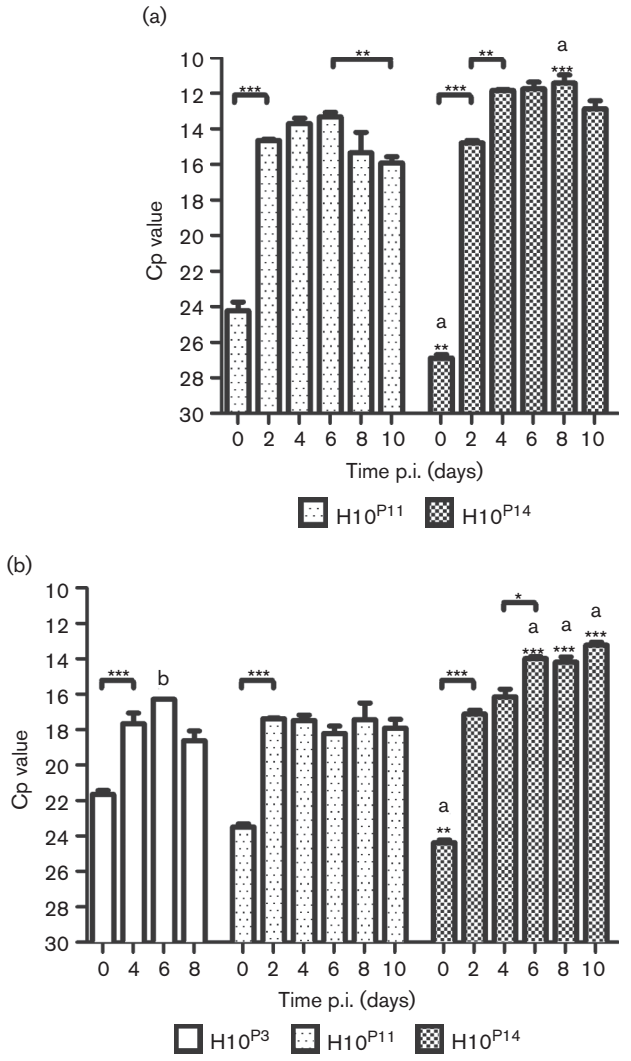

Fig. 4. Viral replication of the three SAV3-H10 passage isolates in CHSE and AGK cell lines. The viral load in triplicate wells was measured by real-time PCR and given as mean Cp-values with SEM. (a) $H10^{P11}$ and $H10^{P14}$ in CHSE cell line. (b) $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$ in AGK cell line. Relevant *P* values are indicated with brackets (***$P<0.001$, **$P<0.01$, *$P<0.05$). a, $H10^{P14}$ *P* value indication to $H10^{P11}$ and $H10^{P3}$ on the equivalent days p.i. (AGK 0 days p.i., only significant difference between $H10^{P3}$ and $H10^{P14}$); b, only one well.

(Fig. 4b). $H10^{P14}$ replicates to higher copy numbers than the two other strains (25-fold higher than $H10^{P11}$ at day 10), which corresponds well with cell viability data (Fig. 3b). Further, there was no significant difference between $H10^{P3}$ and $H10^{P11}$ (in AGK cells) at days 4 and 8 (day 6 included only one well and cannot be used in statistical analysis). When replication was studied in AGK, from which $H10^{P14}$ was derived, $H10^{P14}$ resulted in higher viral titres compared with the CHSE-derived $H10^{P11}$ and $H10^{P3}$ (Fig. 4b). $H10^{P3}$ was not available in sufficiently high titre to allow a full set up (see Methods) and results were thus not included in the statistical comparison on days 2, 6 and 10.

The AGK-grown isolate's high virulence and replication capacity *in vitro* are reversed when tested *in vivo*

SAV infection results in pathological changes in heart, pancreas and skeletal muscle, but mortality and clinical signs might not be present (McLoughlin & Graham, 2007). The highest viral load is found in the heart and pancreas (Andersen *et al.*, 2007; Xu *et al.*, 2012). To compare viral infection levels and corresponding pathological changes, 50 Atlantic salmon parr were infected by intramuscular injection with $10^{5.17}$ $TCID_{50}$ of $H10^{P3}$ and in a parallel tank a similar infection was performed using $H10^{P14}$ at the same infection dose. Sampling was conducted from five non-infected control fish at the day of challenge and from 10 fish per infected tank at 1, 2, 3, 4 and 5 weeks post-challenge. $H10^{P3}$ resulted in a significantly higher viral load in heart tissue than did $H10^{P14}$ at weeks 1–3 post-challenge (Fig. 5a). The trend continued at weeks 4 and 5, but differences in viral load were not significant ($P=0.5678$ and $P=0.1883$). A similar trend was seen in head kidney samples (results not shown) and excluded that the difference between $H10^{P3}$ and $H10^{P14}$ was due to a change in cell tropism. The histology scores for pancreas corresponded with viral load in heart. $H10^{P3}$ infection resulted in significantly higher scores than $H10^{P14}$ at weeks 2 and 5 (Fig. 5b), showing that $H10^{P3}$ has higher virulence and replication capacity compared with reduction for $H10^{P14}$ *in vivo*. For the histology scores in heart tissue, the same trend was observed, but the only statistically significant difference was found at week 2 (Fig. 5c). Cell culture adaptation of SAV3 thus results in loss of *in vivo* virulence.

AGK-grown SAV has increased divergence in structural proteins and a deletion in the 3′UTR

A full-genome sequencing of the H10 plaque-purified isolates was performed on a single clone from strains $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$. In the two ORFs, a total of four separate nucleotide substitutions were found, resulting in divergence at four amino acid positions (Fig. 6a). Sequencing covering these positions, on supernatant material from the serial passages in CHSE and AGK, however, showed low presence of all the diverging amino acid variants and in general the amino acids E1 81G, E1 441L, E2 133Q and nsP1 183D were found. The only exception was the E1 position 441 L to F substitution (L441F) present in the $H10^{P14}$ isolate, which was present in the CHSE-grown $H10^{P2}$ and AGK-grown $H10^{P12}$ supernatants; in AGK-grown $H10^{P13}$, a double peak in the sequencing chromatogram of one nucleotide of the codon was seen, resulting in either L or F. Sequencing of plaque-purified isolates parallel to the $H10^{P14}$ used in the study confirmed that E1 L441F is representative of this passage as eight of ten additional isolates examined had the L441F. Similarly, all

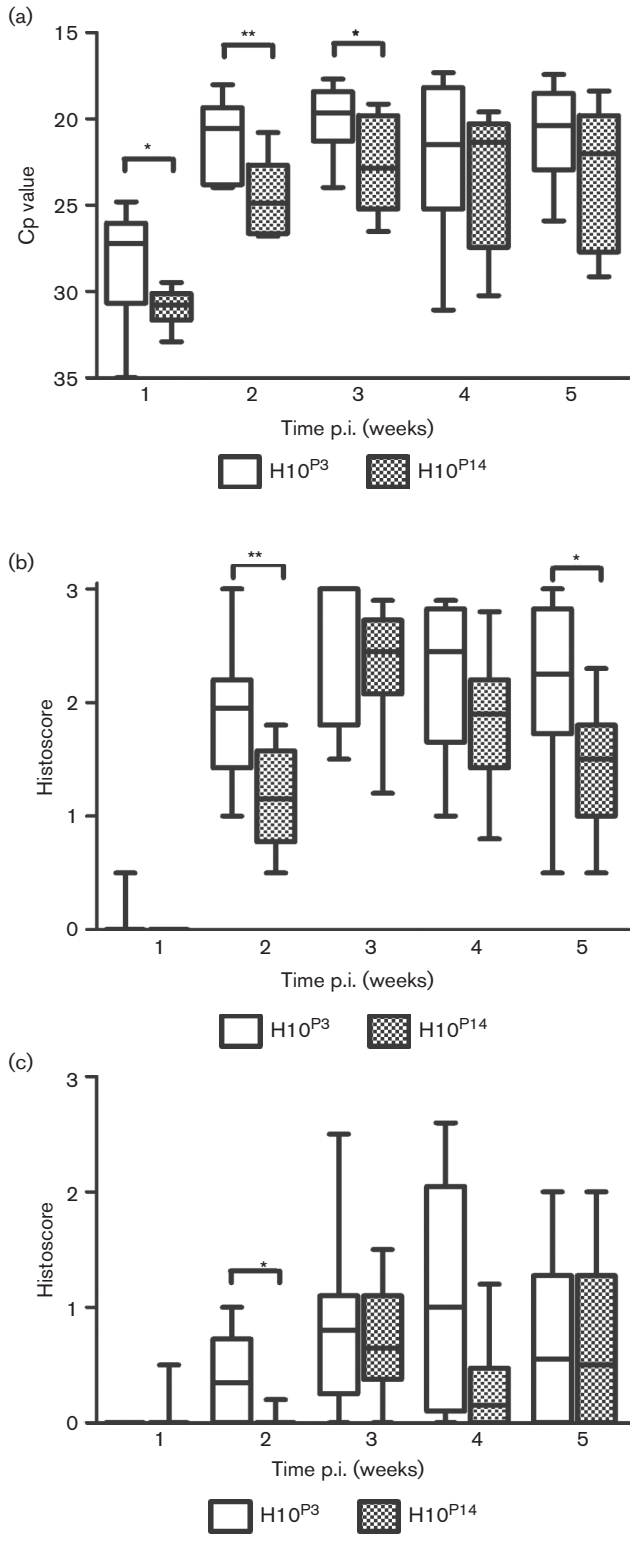

Fig. 5. *In vivo* study. Sequential development of the viral load in heart (a) and histology scores in pancreas (b) and heart (c) following experimental challenge of Atlantic salmon using SAV3 $H10^{P3}$ and $H10^{P14}$, $n=10$. The viral load was measured by real-time PCR and given as Cp values. The results are presented as a box plot (whiskers and minimum to maximum). *$P<0.05$ **$P<0.01$.

ten isolates had E2 Q133K substitutions, with no mutations in the two other positions shown (Fig. 6b). The sequencing also showed that the two mutations found in the purified isolates $H10^{P3}$ and $H10^{P11}$ were not typical representatives of the general virus population in supernatants from the serial passage, i.e. E1 G81D and nsP1 D183N were not found in sequences from serial passage supernatants (Fig. 6b). This was also confirmed by sequencing parallel plaque-purified isolates, which also did not show these mutations.

The UTRs were identical for the 5′ end and for the internal UTR for all three passage isolates. However, the 3′UTR of $H10^{P14}$ contained a 3 nt deletion at positions of 66 to 68 as compared with $H10^{P3}$ and $H10^{P11}$ (Fig. 6c).

DISCUSSION

This study describes the differences in *in vitro* and *in vivo* characteristics of three SAV3-H10 isolates obtained by passage in CHSE-214 followed by AGK cells. An early ($H10^{P3}$) and a later ($H10^{P11}$) passage isolate obtained after three and 11 passages on CHSE-214 were characterized by *in vitro* studies together with an isolate ($H10^{P14}$) obtained from nine passages on CHSE followed by five passages on AGK. *In vivo* pathogenicity of the early CHSE isolate and the AGK-transferred isolate ($H10^{P14}$) was compared by challenge of Atlantic salmon parr. Full-length genome sequences of one single clone of each of the three passage isolates were obtained.

In vitro the AGK-grown isolate showed earlier appearance of CPE, rapid decline in cell viability and increased replication levels compared with the $H10^{P3}$ and $H10^{P11}$ isolates, typical of an adaptation to cell culture. *In vitro* adaptation resulted in delayed onset of pathology in target organs *in vivo*, correlating with lower virus replication levels in the same internal organs. Variation was found at four amino acid positions when comparing the three isolates. $H10^{P14}$ had unique amino acids at two of the four positions and a 3 nt deletion in the 3′UTR compared with $H10^{P3}$ and $H10^{P11}$ isolates.

Previous studies addressing genetic variation of SAV3 isolates have identified several positions of variation at the amino acid level. The majority of available SAV3 WT genomic sequences are from the E2 glycoprotein, and this is also the protein showing the highest variability (Jansen *et al.*, 2010; Petterson *et al.*, 2013). Fourteen amino acid residues of the E2 protein have been found to vary between isolates from the field (Jansen *et al.*, 2010; Karlsen *et al.*, 2006; Petterson *et al.*, 2013). Among these, positions 204 and 206 of the E2 are the only residues presented in more than one study (Jansen *et al.*, 2010; Karlsen *et al.*, 2006, 2014). Our study does not confirm *in vitro* adaptation of E2 S206P (Karlsen *et al.*, 2015), which might be due to different laboratory virus strains resulting from different adaptation to environmental selection pressure using different cell lines. Similar results are seen for the prototypic Old World

(a)

	Protein aa position			
Plaque-purified isolate	**E1 81**	**E1 441**	**E2 133**	**NsP1 183**
$H10^{P3}$	D	L	Q	D
$H10^{P11}$	G	L	Q	N
$H10^{P14}$	G	F	K	D

(b)

	Protein aa position			
Supernatant from serial passages	**E1 81**	**E1 441**	**E2 133**	**NsP1 183**
$H10^{P1–P15}$ in CHSE	G	L (F^{P2})	Q	D
$H10^{P10–P13}$ in AGK	G	L/F[a]	Q	D

Plaque-purified isolate	**E1 81**	**E1 441**	**E2 133**	**NsP1 183**
$H10^{P3}$ isolate 2 and 3	G	L	Q	D
$H10^{P11}$ isolate 2-7	G	L	Q	D
$H10^{P14}$ isolate 2-11	G	F/L[b]	K	D

[a]P10/P11 L only, P12 F only, P13 mix of L and F
[b]L in two of 10 isolates

(c)

```
          1                                                        50
H10P3     AUUCCGGUAUAUAAAUUGCUCACUAGGAGCCCAUCCGAACCCACAGGGAG
H10P11    AUUCCGGUAUAUAAAUUGCUCACUAGGAGCCCAUCCGAACCCACAGGGAG
H10P14    AUUCCGGUAUAUAAAUUGCUCACUAGGAGCCCAUCCGAACCCACAGGGAG

          51                                                91
H10P3     UAGGAUGAGUCAUCUAUUGGUUUUAAAAUUUUCAAUAUAAG3′polyA
H10P11    UAGGAUGAGUCAUCUAUUGGUUUUAAAAUUUUCAAUAUAAG3′polyA
H10P14    UAGGAUGAGUCAUCU---GGUUUUAAAAUUUUCAAUAUAAG3′polyA
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Fig. 6. Divergence in protein sequences and 3′UTR of genomes among the three SAV3 H10 isolates. (a) Protein positions with divergence in amino acids for plaque-purified isolates found after full genome sequencing. (b) Presence of mutations in supernatants from serial passage of SAV3 H10 in CHSE and AGK cell cultures and parallel plaque-purified isolates after control sequencing on PCR products. (c) Alignment of full 3′UTR from first nucleotide after stop codon of structural protein ORF to 3′ poly(A), including sequence homologous to the alphavirus 19nt CSE (underlined).

alphavirus, Sindbis virus, which for many years gave conflicting results on receptor identification that were later explained by adaptation to growth in cell culture (Klimstra *et al.*, 1998, 1999). The E2 glycoprotein constitutes together with the E1 glycoprotein the heterodimer spikes in the membrane of the virus (Cheng *et al.*, 1995; Strauss & Strauss, 1994). E2 forms the distal part of the spikes and is believed to mediate attachment to the host cell, and substitutions in this protein are more likely involved in cell receptor binding. E1 is responsible for fusion between viral and host endosomal membranes (Omar & Koblet, 1988; Pletnev *et al.*, 2001). Substitutions in positions involved in receptor binding are probably more important than positions involved in membrane fusion when it comes to adaptation of the virus to a new host, since the variability in cell surface molecules is higher than that in intracellular membrane components. It is therefore tempting to speculate that the E2 Q133K substitution plays a role in this regard. The substitution is also interesting as it results in a change from an amino acid with a neutral

side-chain charge to a side chain with positive charge. For alphaviruses, it has been shown that increased affinity for heparan sulfate as receptor *in vitro* is based on a change towards more positively charged residues in the receptor binding region (Bernard *et al.*, 2000; Byrnes & Griffin, 1998; Gardner *et al.*, 2014; Heil *et al.*, 2001). However, when virulence was tested *in vivo* it was generally decreased, which suggests adaptation of the virus *in vitro* (Byrnes & Griffin, 2000; Klimstra *et al.*, 1999). Still, the change in virulence observed for the adapted virus is likely multifactorial and L441F might also play a role. The substitutions seen in E1 position 81 and nsP1 position 183 between $H10^{P3}$ and $H10^{P11}$ are probably of lesser importance as they were not found in any of the virus supernatants from the serial passaging or in any of the other plaque-purified isolates tested. Still, the results available for comparison of $H10^{P3}$ against $H10^{P11}$ indicate a slightly higher virulence for $H10^{P11}$ when grown in CHSE cells and some relevance of the mutations cannot be ruled out. The study of viral replication showed that although the same m.o.i. was used for all the isolates, a significantly higher viral load was cell-associated at 0 days p.i. when the inoculum was removed at 4 h of incubation, for $H10^{P11}$ versus $H10^{P14}$ in CHSE and similarly $H10^{P3}$ versus $H10^{P14}$ in AGK. Although $H10^{P14}$ had less virus associated with the cells, this isolate showed the highest viral loads in both cell lines p.i., which points towards replication efficiency being more important than cell receptor binding per se.

The 3 nt deletion in the 3′UTR of the late, AGK-grown isolate ($H10^{P14}$) is interesting. The 3′UTR of alphaviruses and of other positive sense RNA-viruses is believed to be important in regulating minus-strand RNA synthesis (Hardy & Rice, 2005; Kuhn *et al.*, 1990). The last 19 nt prior to the poly(A) on the genome are highly conserved among alphaviruses and of special importance to the minus-strand RNA synthesis. The full 3′UTR is highly conserved among salmonid alphaviruses both in length and nucleotide sequence, but the 3′ end shows significant divergence in the 19 nt conserved sequence element (3′CSE) preceding the poly(A) when compared with those of other alphaviruses (Weston *et al.*, 1999). The 3 nt deletion found in $H10^{P14}$ is located in the region that putatively holds a function similar to other alphavirus 3′CSEs (Karlsen *et al.*, 2009; Weston *et al.*, 1999). It is tempting to suggest that changes in the 3′UTR region are a result of adaptation to cell culture conditions of AGK cells and that they facilitate genome replication. Obviously, additional studies, including reverse genetic methods, would have to be employed to better understand the underlying mechanisms. *In silico* predictions show that the deletion results in a pronounced change in the predicted secondary structure of the 3′UTR of $H10^{P14}$ compared with that of $H10^{P3}$ and $H10^{P11}$ (not shown) and it would be interesting to include studies on the importance of the 3′UTR secondary structure in future studies of genome replication of SAV3.

In vitro passaging of SAV3-H10 showed adaptation to cell culture and resulted in an increased replication rate, earlier CPE in infected cells and earlier decline in cell viability. In detail, measurements of viral replication in both cell lines showed that the AGK-transferred isolate ($H10^{P14}$) had significantly higher replication than the two earlier CHSE-cultured isolates, after infection of AGK cells and also in CHSE cells, although the evidence in the available data material was less significant in this cell line. Both cell morphology and viability data confirmed this trend. Neither the cell viability study nor the study of viral replication showed any differences between $H10^{P3}$ and $H10^{P11}$, both passaged on CHSE only. This indicates that the adaptation resulting from transfer to and passage in AGK cells is of higher impact than the passage length in CHSE cells in this study. This is also supported by the more pronounced differences in the assays on AGK cells than in the assays on CHSE cells, between the two CHSE-only grown isolates compared with the AGK transferred isolate. *In vivo*, the consequence of adaptation to cell culture was reduced virus replication and reduced tissue damage in the target host. Viral load in heart was lowest for the AGK-grown isolate ($H10^{P14}$) throughout the challenge period, and tissue pathology by histology was also lower for this isolate compared with the CHSE-grown isolate. The viral load measured in heart tissue and the tissue pathology were clearly related in our study, where the pathological changes in pancreas increased with viral load in the heart. The relationship between viral load and tissue pathology for SAV3 has been documented earlier, and a threshold of virus replication has to be reached to trigger pathology (Xu *et al.*, 2012). Difference in tissue tropism between the two passage isolates in our study was less likely since the viral load in head kidney followed the same pattern as heart tissue (results not shown). This is not surprising since it has been found for other alphaviruses that the attenuation is due to slower spread and decreased destruction rather than to differences in cell tropism (Fazakerley *et al.*, 1992).

We showed that SAV3-H10 as a result of cell culture passage loses virulence *in vivo*, i.e. ability to induce pathology. Attenuation of arthropod-borne alphaviruses is well known to occur through blind cell culture passage and has been useful in the development of live vaccine strains (Gardner *et al.*, 2014; McKinney *et al.*, 1963; Roukens & Visser, 2008). The molecular mechanism of attenuation using such approaches is, however, poorly understood. Attenuation *in vivo* has been correlated with selection of rapid penetration of cells *in vitro* for several alphaviruses (Davis *et al.*, 1991; Glasgow *et al.*, 1991; Russell *et al.*, 1989). Some studies have pinpointed the major amino acid determinant of attenuation, for Venezuelan equine encephalitis virus E2 amino acid 120 (Kinney *et al.*, 1993) interestingly located in the same sequence region as our E2 Q133K. To what extent further passage *in vitro* of SAV3-H10 would result in attenuation to a degree it can serve as a vaccine strain remains to be shown. Also the reversion virulence is an issue that would have to receive careful attention. Our results show that AGK cell-culture adaptation leads to lesser virulence *in vivo* and are in conformity

with previous findings in alphaviruses (in general), and the histology is consistent with previously published studies on SAV (Xu *et al.*, 2012). A recent study has shown that strains of SAV3 differ in virulence (Taksdal *et al.*, 2014); however, no attempt was made to elucidate underlying virulence motifs or differences between strains at the genetic level. It is likely that the studies performed herein better serve as an approach for understanding the underlying patterns of virulence than for pointing out the direction for an attenuated vaccine virus. Thus, future work should include determination of the residues/combination of residues defining SAV3 virulence. The approach should include reverse genetics but has to be guided by *in vivo* results, which could also include passage of $H10^{P14}$ in fish to study the possibility of reversion of the cell-culture-adapted mutations and subsequent virulence *in vivo*. Field data, which so far have been scant, should also be included.

METHODS

Cells and viruses. Chinook salmon embryo cells (CHSE-214; ATCC CRL-1681) were maintained at 20 °C in Leibovitz's L-15 medium with GlutaMAX (Invitrogen) supplemented with 5% FBS and 50 µg gentamicin ml^{-1}. Asian Grouper strain K (AGK) (Munang'andu *et al.*, 2012) cells were maintained at 28 °C in the same L-15 medium supplemented with 7.5% FBS. Virus-inoculated cells were incubated at 15 °C in L-15 medium supplemented with 2% FBS and 50 µg gentamicin ml^{-1} for both cell lines and these conditions were also used in the following studies unless otherwise stated. The SAV3 isolate used in this study originates from heart tissue from a fish diagnosed with PD as described by Guo *et al.* (2014) and Xu *et al.* (2010). Homogenate supernatant was used for initial inoculation and further propagated by serial passages until the 11th passage in the CHSE cell line. A fixed ratio of supernatant volume per cell culture was used with minor adjustments along the serial passage. The first five passages were incubated for 10–14 days and passaged when the development of a weak CPE terminated. From passage five, each passage was harvested after 7–10 days p.i. due to a faster developing/increased degree of final CPE. The 9th passage was further inoculated on AGK cells and passaged an additional five times (p9 to p14), also with harvest at 7–10 days post-inoculation. At passage, virus supernatant was harvested by one freeze–thaw cycle unless full CPE was observed. Virus supernatant was clarified by low speed centrifugation before transferring to a naïve cell culture. Virus supernatant from an early passage on CHSE (3rd) referred to as $H10^{P3}$, one later passage (11th on CHSE) referred to as $H10^{P11}$ and one from a passage obtained from nine passages on CHSE followed by five passages on AGK referred to as $H10^{P14}$, were selected for further studies (Fig. 1).

Plaque purification of virus. Virus from the supernatants of the three selected passages was plaque-purified before full-length genome sequencing and experimental use. Briefly, confluent CHSE cells in 0.3 ml L-15 medium in six-well plates were inoculated with 0.1 ml 10-fold dilutions (10^{-1}–10^{-6}) of cell culture supernatants from passages $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$ and incubated for 4.5 h at 15 °C. Subsequently, the inoculum was removed and the cells were overlaid with 2 ml of L-15 medium with 5% FBS, 2% L-glutamine, 50 µg gentamicin ml^{-1} and 0.8% SeaPlaque agarose (Lonza). After agarose gelling at room temperature, the cells were incubated at 15 °C for four days. Plaques were picked for each passage isolate by gel punch biopsy while the cells were visualized by microscopy, and used directly for inoculation onto CHSE cells in 25 cm^2 flasks and subsequently passaged three times using conditions as described earlier for virus-inoculated cells. When CPE was evident, cells and supernatant were frozen and thawed before further passaging and the supernatant was centrifuged to remove cell debris. The infectious titres of the final supernatants were determined by end-point dilution on CHSE cells grown in 96-well plates, estimating the $TCID_{50}$ by the method of Kärber (1931), prior to cell infection assays and fish challenge (described below). Titration after plaque purification showed that $H10^{P14}$ resulted in the highest titre ($10^{7.8}$ $TCID_{50}$ ml^{-1}) and $H10^{P11}$ (10^{7} $TCID_{50}$ ml^{-1}) and $H10^{P3}$ the lowest ($10^{6.8}$ $TCID_{50}$ ml^{-1}).

Time-course observation of morphological changes in cell culture. CHSE and AGK cells were grown in 96-well plates until 80% confluence and infected in triplicate with plaque-purified $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$ passage isolates at m.o.i. 10 and further grown in L-15 medium as described for infected cells above. Non-infected cells were kept under the same conditions and used as a negative control. Cellular morphology and signs and degree of CPE were documented using phase-contrast microscopy (Olympus) using 10x magnification objective on all images, at 0, 12 and 24 h and 2, 4, 6, 7, 8, 9, 10, 12 and 14 days p.i.

Cell viability assay. CHSE and AGK were prepared and infected as described for the morphological observations above using the three plaque-purified passage isolates at m.o.i. 10 in quadruplicate wells. Similarly, non-infected cells were used as a control. The viability of the cells was analysed at 0, 2, 4, 5, 6, 7, 8 and 10 days p.i. using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (MTS) (Promega), according to the manufacturer's procedures, with minor adjustments as the cells were incubated at 15 °C for 24 h after addition of the CellTiter 96 AQ_{ueous} One Solution reagent. The quantity of resulting formazan product was measured using colorimetry by scanning multiwell spectrophotometry (Tecan GENios) at 490 nm. An increase in the number of viable and metabolically active cells results in an increase in formazan product.

Virus replication kinetics in cells measured by real-time PCR. Cells were infected with plaque-purified $H10^{P11}$ and $H10^{P14}$ at m.o.i. 10 in triplicate on 24-well plates of CHSE and AGK cell lines. $H10^{P3}$ was also inoculated in a reduced set up in the AGK cell line only, due to low titre and limited availability of inoculum of this passage isolate. Inoculum was added to the cells together with L-15 medium before incubation at 15 °C for 4 h. After incubation, the inoculum was removed and the wells were washed once with PBS before adding fresh L-15 medium and incubation continued. Sampling was performed after removal of inoculum and washing (0 days p.i.) and at 2, 4, 6, 8 and 10 days p.i. for $H10^{P11}$ and $H10^{P14}$, and at 0, 2, 4, 6 (one parallel only) and 8 days p.i. for $H10^{P3}$. RNA was extracted from the cells using an RNeasy mini kit (Qiagen) according to the protocol of the manufacturer. After removal of the supernatant, the cells were lysed in 350 µl RLT buffer and homogenized using QIAshredder columns at every sampling and the lysates were stored at −80 °C until further RNA isolation. RNA was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). cDNA synthesis was performed using a SuperscriptIII reverse transcriptase kit (Invitrogen), 165 ng RNA and random hexamers priming the reaction. Subsequent real-time PCR analysis was performed using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) as described previously (Petterson *et al.*, 2013), using primers against the E2 gene. The reactions were run in duplicate using 2 µl undiluted cDNA as template in a 20 µl reaction.

***In vivo* challenge.** A challenge study was conducted at the aquarium facilities of the Norwegian University of Life Science/Norwegian Veterinary Institute in Oslo, Norway. The experiment was approved by the Norwegian Animal Research Authority and the local IACUC of the Norwegian University of Life Sciences. A total of 110 Atlantic salmon (*S. salar* L.) parr, with an average mass of 80 g, were included

in the study. The fish were randomly divided with 50 parr to each of two tanks and anaesthetized in 30–40 mg l^{-1} benzocain, weighed and subsequently challenged with $H10^{P3}$ and $H10^{P14}$, respectively, by intramuscular injection of 100 µl supernatant from infected cell cultures ($10^{6.17}$ $TCID_{50}$ ml^{-1} for both passage isolates). A third tank with 10 non-treated parr was used as negative control. Fresh water with temperature 12 ± 1 °C was used in the tanks. Organ sampling of 10 fish per tank was conducted 1, 2, 3, 4 and 5 weeks post-challenge (p.c.). At 0 and 5 weeks p.c., five fish were sampled from the negative control tank. The fish were sedated with approximately 17 mg l^{-1} AQUI-S and euthanasia was performed by a sharp blow to the head before heart and head kidney tissue were sampled and preserved in RNAlater (Invitrogen). Samples were stored at −20 °C until RNA extraction was performed. Parallel samples from heart, pancreas and skeletal muscle were submerged in 10% phosphate-buffered formalin and processed for paraffin-embedment and sectioning. Tissue specimens were stained with haematoxylin and eosin, all using standard methods. Evaluation of histopathological changes in heart, pancreas and skeletal muscles was performed as described by Xu *et al.* (2012). In brief, scoring was made on a visual analogue scale between 0 and 3 for both heart and pancreas, with pancreas changes starting as focal necrosis and moderate inflammation (score 1) and extending to diffuse changes with complete loss of exocrine pancreas (score 3). For heart, mild changes were focal infiltration with inflammatory cells and individual necrosis of cardiomyocytes (score 1) and severe cases showed diffuse necrosis of cardiomyocytes and intense infiltration of inflammatory cells (score 3).

RNA was extracted from heart and kidney tissue using the RNeasy Fibrous tissue mini kit and RNeasy mini kit (Qiagen), respectively, according to the kit protocols. The tissue was homogenized in RLT buffer with β-mercaptoethanol using steel beads in a mixer mill MM301 (Retsch) for 2 min at 20 Hz. RNA quantification and cDNA synthesis was performed as described above using 1.1 µg RNA for heart samples and 220 ng RNA for kidney samples in the reaction. Real-time PCR analysis was conducted with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) using 2 µl 1:2 diluted cDNA as template as described previously (Petterson *et al.*, 2013).

Full-length viral genome sequencing. $H10^{P11}$ has previously been subjected to full-length genome sequencing as described in an earlier study where an infectious SAV3 cDNA clone was constructed using $H10^{P11}$ genome sequence as the cDNA template (Guo *et al.*, 2014; SAV3-H10 GenBank accession no. JQ799139.1) and $H10^{P3}$ and $H10^{P14}$ were full-length genome sequenced using the same procedures. In brief, plaque purified supernatant was centrifuged to remove cell debris before virus sedimentation was performed by ultracentrifugation (Beckman Optima Ultracentrifuge with rotor TLA-45) at 65000 ***g*** for 1.5 h. RNA isolation and cDNA synthesis and subsequent PCR using DNA polymerase with proofreading capabilities were performed using commercial kits. PCR amplification of the full genome was performed by amplification of two fragments of approximately 6.5 kb and 5.5 kb, flanked with 5′ *Eco*RI and 3′ *Afl*II/*Not*I restriction sites and 5′ *Eco*RI/*Afl*II and 3′ *Not*I, respectively, for the two fragments. PCR products were separated in a 0.8% GTG gel and purified using a Qiagen gel extraction kit. Both amplified fragments were cloned separately into *Eco*RI and *Not*I sites of the pCR2.1 vector (Invitrogen) following standard cloning procedures, before transformation into competent OneShot TOP10 bacterial cells (Invitrogen) according to manufacturer's procedures. Positive clones were confirmed by PCR methods using specific primers for each fragment and cultivated in LB medium with 100 mg ampicillin l^{-1}. After plasmid purification, using a commercial kit, the two vectors containing 6.5 kb and 5.5 kb fragments were digested with *Afl*II and *Not*I and the linearized plasmid containing the pCR2.1 backbone and 6.5 kb fragment and the separated 5.5 kb insert, respectively, were visualized by gel electrophoresis and purified as described previously. The fragments were combined by subcloning the 5.5 kb fragment into the vector containing the 6.5 kb fragment by ligation using the *Afl*II/*Not*I sites, resulting in a plasmid with a full-length genome of the virus. Full-length sequences of $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$ were obtained from purified plasmid of a single clone using 20 forward primers resulting in overlapping sequences. Primers were designed using Vector NTI advance 11.0 software (Invitrogen) (primers not shown) and sequencing performed using a commercial service (Eurofins MWG operon or GATC biotech). The full-length sequences for $H10^{P3}$, $H10^{P11}$ and $H10^{P14}$ were aligned in Contig Express of Vector NTI advance 11.0.

To verify if amino acid mutations of the plaque-purified isolates used for *in vitro* and *in vivo* testing were representative of the virus from the serial passage in CHSE and AGK cells, sequencing of PCR products was done covering the regions with observed mutations. This was done from amplified RNA isolated from the supernatants in the serial passages using standard procedures and commercial sequencing services. Similar sequencing was also performed on supernatant material from the three isolates used in the study and on several parallel plaque purified isolates.

Statistical methods. The statistical calculations were performed using GraphPad Prism version 5.0 and 6.0. A Two-Way Anova with Tukey's multiple comparisons test with alpha level 0.05 was performed on cell proliferation data, and One-Way Anova with Tukey's multiple comparisons test was performed on viral replication data. In the *in vivo* study, a two-tailed Mann–Whitney test was performed on both viral load and histological scores.

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REFERENCES

Andersen, L., Bratland, A., Hodneland, K. & Nylund, A. (2007). Tissue tropism of salmonid alphaviruses (subtypes SAV1 and SAV3) in experimentally challenged Atlantic salmon (*Salmo salar* L.). *Arch Virol* **152**, 1871–1883.

Bernard, K. A., Klimstra, W. B. & Johnston, R. E. (2000). Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. *Virology* **276**, 93–103.

Byrnes, A. P. & Griffin, D. E. (1998). Binding of Sindbis virus to cell surface heparan sulfate. *J Virol* **72**, 7349–7356.

Byrnes, A. P. & Griffin, D. E. (2000). Large-plaque mutants of Sindbis virus show reduced binding to heparan sulfate, heightened viremia, and slower clearance from the circulation. *J Virol* **74**, 644–651.

Castric, J., Baudin-Laurencin, F., Brèmont, M., Le Ven, A. & Bèarzotti, M. (1997). Isolation of the virus responsible for sleeping-disease in experimentally infected rainbow-trout *Oncorhynchus mykiss*. *Bull Eur Assoc Fish Pathol* **17**, 27–30.

Chen, L., Evensen, Ø. & Mutoloki, S. (2014). Delayed protein shut down and cytopathic changes lead to high yields of infectious pancreatic necrosis virus cultured in Asian Grouper cells. *J Virol Methods* **195**, 228–235.

Cheng, R. H., Kuhn, R. J., Olson, N. H., Rossmann, M. G., Choi, H. K., Smith, T. J. & Baker, T. S. (1995). Nucleocapsid and glycoprotein organization in an enveloped virus. *Cell* **80**, 621–630.

Davis, N. L., Powell, N., Greenwald, G. F., Willis, L. V., Johnson, B. J., Smith, J. F. & Johnston, R. E. (1991). Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: construction of single and multiple mutants in a full-length cDNA clone. *Virology* **183**, 20–31.

Fazakerley, J. K., Parker, S. E., Bloom, F. & Buchmeier, M. J. (1992). The V5A13.1 envelope glycoprotein deletion mutant of mouse hepatitis virus type-4 is neuroattenuated by its reduced rate of spread in the central nervous system. *Virology* **187**, 178–188.

Fringuelli, E., Rowley, H. M., Wilson, J. C., Hunter, R., Rodger, H. & Graham, D. A. (2008). Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. *J Fish Dis* **31**, 811–823.

Gardner, C. L., Hritz, J., Sun, C., Vanlandingham, D. L., Song, T. Y., Ghedin, E., Higgs, S., Klimstra, W. B. & Ryman, K. D. (2014). Deliberate attenuation of chikungunya virus by adaptation to heparan sulfate-dependent infectivity: a model for rational arboviral vaccine design. *PLoS Negl Trop Dis* **8**, e2719.

Glasgow, G. M., Sheahan, B. J., Atkins, G. J., Wahlberg, J. M., Salminen, A. & Liljeström, P. (1991). Two mutations in the envelope glycoprotein E2 of Semliki Forest virus affecting the maturation and entry patterns of the virus alter pathogenicity for mice. *Virology* **185**, 741–748.

Guo, T. C., Johansson, D. X., Haugland, Ø., Liljeström, P. & Evensen, Ø. (2014). A 6K-deletion variant of salmonid alphavirus is non-viable but can be rescued through RNA recombination. *PLoS One* **9**, e100184.

Hardy, R. W. & Rice, C. M. (2005). Requirements at the 3′ end of the *Sindbis virus* genome for efficient synthesis of minus-strand RNA. *J Virol* **79**, 4630–4639.

Heil, M. L., Albee, A., Strauss, J. H. & Kuhn, R. J. (2001). An amino acid substitution in the coding region of the E2 glycoprotein adapts Ross River virus to utilize heparan sulfate as an attachment moiety. *J Virol* **75**, 6303–6309.

Hjortaas, M. J., Skjelstad, H. R., Taksdal, T., Olsen, A. B., Johansen, R., Bang-Jensen, B., Ørpetveit, I. & Sindre, H. (2013). The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, *Salmo salar* L., in Norway. *J Fish Dis* **36**, 71–74.

Hodneland, K., Bratland, A., Christie, K. E., Endresen, C. & 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.

Jansen, M. D., Gjerset, B., Modahl, I. & Bohlin, J. (2010). Molecular epidemiology of salmonid alphavirus (SAV) subtype 3 in Norway. *Virol J* **7**, 188.

Kärber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch Exp Pathol Pharmakol* **162**, 480–483.

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

Karlsen, M., Villoing, S., Rimstad, E. & Nylund, A. (2009). Characterization of untranslated regions of the salmonid alphavirus 3 (SAV3) genome and construction of a SAV3 based replicon. *Virol J* **6**, 173.

Karlsen, M., Gjerset, B., Hansen, T. & Rambaut, A. (2014). Multiple introductions of salmonid alphavirus from a wild reservoir have caused independent and self-sustainable epizootics in aquaculture. *J Gen Virol* **95**, 52–59.

Karlsen, M., Andersen, L., Blindheim, S. H., Rimstad, E. & Nylund, A. (2015). A naturally occurring substitution in the E2 protein of Salmonid alphavirus subtype 3 changes viral fitness. *Virus Res* **196**, 79–86.

Kinney, R. M., Chang, G. J., Tsuchiya, K. R., Sneider, J. M., Roehrig, J. T., Woodward, T. M. & Trent, D. W. (1993). Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5′-noncoding region and the E2 envelope glycoprotein. *J Virol* **67**, 1269–1277.

Klimstra, W. B., Ryman, K. D. & Johnston, R. E. (1998). Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *J Virol* **72**, 7357–7366.

Klimstra, W. B., Ryman, K. D., Bernard, K. A., Nguyen, K. B., Biron, C. A. & Johnston, R. E. (1999). Infection of neonatal mice with Sindbis virus results in a systemic inflammatory response syndrome. *J Virol* **73**, 10387–10398.

Kuhn, R. J., Hong, Z. & Strauss, J. H. (1990). Mutagenesis of the 3′ nontranslated region of Sindbis virus RNA. *J Virol* **64**, 1465–1476.

Ludwig, G. V., Kondig, J. P. & Smith, J. F. (1996). A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. *J Virol* **70**, 5592–5599.

McKinney, R. W., Berge, T. O., Sawyer, W. D., Tigertt, W. D. & Crozier, D. (1963). Use of an attenuated strain of Venezuelan equine encephalomyelitis virus for immunization in man. *Am J Trop Med Hyg* **12**, 597–603.

McLoughlin, M. F. & Graham, D. A. (2007). Alphavirus infections in salmonids—a review. *J Fish Dis* **30**, 511–531.

McLoughlin, M. F., Nelson, R. T., Rowley, H. M., Cox, D. I. & Grant, A. N. (1996). Experimental pancreas disease in Atlantic salmon *Salmo salar* post-smolts induced by salmon pancreas disease virus (SPDV). *Dis Aquat Organ* **26**, 117–124.

McLoughlin, M. F., Graham, D. A., Norris, A., Matthews, D., Foyle, L., Rowley, H. M., Jewhurst, H., MacPhee, J. & Todd, D. (2006). Virological, serological and histopathological evaluation of fish strain susceptibility to experimental infection with salmonid alphavirus. *Dis Aquat Organ* **72**, 125–133.

Mcvicar, A. H. (1987). Pancreas disease of farmed Atlantic salmon, *Salmo salar*, in Scotland: epidemiology and early pathology. *Aquaculture* **67**, 71–78.

Mérour, E., Lamoureux, A., Bernard, J., Biacchesi, S. & Brémont, M. (2013). A fully attenuated recombinant Salmonid alphavirus becomes pathogenic through a single amino acid change in the E2 glycoprotein. *J Virol* **87**, 6027–6030.

Munang'andu, H. M., Fredriksen, B. N., Mutoloki, S., Brudeseth, B., Kuo, T. Y., Marjara, I. S., Dalmo, R. A. & Evensen, Ø. (2012). Comparison of vaccine efficacy for different antigen delivery systems for infectious pancreatic necrosis virus vaccines in Atlantic salmon (*Salmo salar* L.) in a cohabitation challenge model. *Vaccine* **30**, 4007–4016.

Nelson, R. T., McLoughlin, M. F., Rowley, H. M., Platten, M. A. & Mccormick, J. I. (1995). Isolation of a toga-like virus from farmed Atlantic salmon *Salmo salar* with pancreas disease. *Dis Aquat Organ* **22**, 25–32.

Omar, A. & Koblet, H. (1988). Semliki Forest virus particles containing only the E1 envelope glycoprotein are infectious and can induce cell-cell fusion. *Virology* **166**, 17–23.

Petterson, E., Stormoen, M., Evensen, Ø., Mikalsen, A. B. & Haugland, Ø. (2013). Natural infection of Atlantic salmon (*Salmo salar* L.) with salmonid alphavirus 3 generates numerous viral deletion mutants. *J Gen Virol* **94**, 1945–1954.

Pletnev, S. V., Zhang, W., Mukhopadhyay, S., Fisher, B. R., Hernandez, R., Brown, D. T., Baker, T. S., Rossmann, M. G. & Kuhn, R. J. (2001). Locations of carbohydrate sites on alphavirus

glycoproteins show that E1 forms an icosahedral scaffold. *Cell* **105**, 127–136.

Roukens, A. H. & Visser, L. G. (2008). Yellow fever vaccine: past, present and future. *Expert Opin Biol Ther* **8**, 1787–1795.

Russell, D. L., Dalrymple, J. M. & Johnston, R. E. (1989). Sindbis virus mutations which coordinately affect glycoprotein processing, penetration, and virulence in mice. *J Virol* **63**, 1619–1629.

Strauss, J. H. & Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58**, 491–562.

Taksdal, T., Bang Jensen, B., Böckerman, I., McLoughlin, M. F., Hjortaas, M. J., Ramstad, A. & Sindre, H. (2014). Mortality and weight loss of Atlantic salmon, *Salmon salar* L., experimentally infected with salmonid alphavirus subtype 2 and subtype 3 isolates from Norway. *J Fish Dis.* [Epub ahead of print].

Welsh, M., Weston, J., Borghmans, B. J., Mackie, D., Rowley, H., Nelson, R., McLoughlin, M. & Todd, D. (2000). Biochemical characterization of salmon pancreas disease virus. *J Gen Virol* **81**, 813–820.

Weston, J. H., Welsh, M. D., McLoughlin, M. F. & Todd, D. (1999). Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, *Salmo salar* L. *Virology* **256**, 188–195.

Weston, J., Villoing, S., Brémont, M., Castric, J., Pfeffer, M., Jewhurst, V., McLoughlin, M., Rødseth, O., Christie, K. E. & other authors (2002). Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. *J Virol* **76**, 6155–6163.

Xu, C., Guo, T. C., Mutoloki, S., Haugland, Ø., Marjara, I. S. & Evensen, Ø. (2010). Alpha interferon and not gamma interferon inhibits salmonid alphavirus subtype 3 replication *in vitro*. *J Virol* **84**, 8903–8912.

Xu, C., Guo, T. C., Mutoloki, S., Haugland, O. & Evensen, O. (2012). Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon. *Vet Res* **43**, 78.

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Natural infection of Atlantic salmon (*Salmo salar* L.) with salmonid alphavirus 3 generates numerous viral deletion mutants

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Salmon pancreas disease virus (SPDV) also referred to as salmonid alphavirus (SAV) is a virus causing pancreas disease in Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*). Although the virus causes an economically important disease, relatively few full-length genome sequences of SAV strains are currently available. Here, we report full-length genome sequences of nine SAV3 strains from sites farming Atlantic salmon geographically spread along the Norwegian coastline. The virus genomes were sequenced directly from infected heart tissue, to avoid culture selection bias. Sequence analysis confirmed a high level of sequence identity within SAV3 strains, with a mean nucleotide diversity of 0.11 %. Sequence divergence was highest in 6K and E2, while lowest in the capsid protein and the non-structural proteins (nsP4 and nsP2). This study reports for the first time that numerous defective viruses containing genome deletions are generated during natural infection with SAV. Deletions occurred in all virus strains and were not distributed randomly throughout the genome but instead tended to aggregate in certain areas. We suggest imprecise homologous recombination as an explanation for generation of defective viruses with genome deletions. The presence of such viruses, provides a possible explanation for the difficulties in isolating SAV in cell culture. Primary virus isolation was successfully achieved for only two of eight strains, despite extensive attempts using three different cell lines. Both SAV isolates were easily propagated further and concomitant viral deletion mutants present in clinically infected heart tissue were maintained following serial passage in CHH-1 cells.

INTRODUCTION

Pancreas disease (PD) is an economically important disease in European salmonid aquaculture affecting both Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) (McLoughlin & Graham, 2007). The disease is characterized by degeneration and necrosis of cardiomyocytes with subsequent inflammation, pancreatic acinar cell loss and subsequent skeletal muscle degeneration (McLoughlin *et al.*, 2006; McVicar, 1987). PD is caused by salmon pancreas disease virus (SPDV) also referred to as salmonid alphavirus (SAV), and belongs to the genus *Alphavirus* within the family *Togaviridae*. The alphaviruses are small, spherical, enveloped viruses that are important pathogens of animals and humans worldwide. Their genomes consist of a monopartite, single stranded, positive-sense RNA genome of 11–12 kb.

Most alphaviruses are transmitted by highly specific arthropod vectors, of which mosquitoes are the most common, but others exist such as lice and mites (Durden *et al.*, 1992). This together with specific environmental conditions and reservoir hosts might lead to restricted geographical distribution (Griffin, 2007). Although SAV3 has been detected by real-time PCR from the salmon louse *Lepeophtheirus salmonis* collected from diseased fish (Petterson *et al.*, 2009), the role of lice or other invertebrate vectors is yet to be determined as SAV infections can be transmitted without the aid of an arthropod (McLoughlin *et al.*, 1996; Xu *et al.*, 2012).

Genetic analysis of salmonid alphaviruses has shown at least six genetically distinct subtypes, SAV1–6, which are also separated geographically (Fringuelli *et al.*, 2008; Graham *et al.*, 2012; Weston *et al.*, 2005). Recently, a comparative experimental study in Atlantic salmon, conducted as a freshwater cohabitation trial, showed that

The GenBank/EMBL/DDBJ accession numbers for the genome sequences of the nine SAV3 strains reported in this paper are KC122918–KC122926.

One supplementary table is available with the online version of this paper.

all subtypes caused pathological changes typical of PD, although the relative virulence of the strains varied (Graham *et al.*, 2011).

SAV3 represents a subtype that so far has been detected only in Norway where it causes PD in Atlantic salmon and rainbow trout (Hodneland *et al.*, 2005). However, following the first detection of a SAV2-related virus in 2011, this subtype is now frequently found in PD outbreaks in mid-Norway (Hjortaas *et al.*, 2013). The common understanding has been that there is little genetic variation within subtypes (Fringuelli *et al.*, 2008; Karlsen *et al.*, 2006; Weston *et al.*, 2005), and previously published sequence data from the Norwegian strains show only minor amino acid differences between the strains analysed (Jansen *et al.*, 2010; Karlsen *et al.*, 2006). However, very few full-length SAV sequences have been published, and the SAV3 full-length sequences previously reported come from the same geographical area, and might therefore not be representative of the entire Norwegian coast. Furthermore, most available full-genome SAV sequences are based on isolates cultured in cells prior to sequencing. As primary virus isolation from field outbreaks has been difficult and yielded relatively few numbers of isolates (McLoughlin & Graham, 2007), the understanding has been that there is need for some degree of selection or adaptation before virus can be isolated in cell culture (Castric *et al.*, 1997; Nelson *et al.*, 1995). A previous study has shown that mutations occur following serial passage in CHSE-214 cells, and four amino acid substitutions were identified in the genome between passage 3 and 20 of one defined isolate of SAV3 (Karlsen *et al.*, 2006).

This paper describes sequence analysis of full-length viral genomes of SAV3 from nine sites geographically spread along the Norwegian coastline. To avoid culture selection bias we performed whole genome sequencing directly from infected fish tissues. The sequence analysis confirmed little divergence between the Norwegian strains, but revealed that natural infection of SAV3 generates numerous viral deletion mutants. Defective interfering virus particles, with deletions in their genome, present in the infected tissues, provide a possible explanation for difficulties regarding primary virus isolation. SAV isolates that were successfully recovered in cell culture were easily propagated subsequently. We further document that defective viruses were maintained following serial passage in CHH-1 cells presumably due to complementation by fully functional genomes.

RESULTS

PD confirmed in Atlantic salmon study samples

Nine Atlantic salmon farms with a previous diagnosis of PD were available and included in the study (Fig. 1). The PD diagnosis was confirmed by histopathological evaluation of haematoxylin and eosin (H/E) stained sections, showing changes typical for PD in heart, pancreas and muscle tissue of the individuals sampled from all locations. The degree of pathological changes ranged from mild to severe and chronic. Presence of SAV in parallel heart tissue was shown by real-time reverse-transcription PCR (RT-PCR) and ranged from 30 % SAV positive individuals in SAV3-2-MR/10 to 80 % in SAV3-1-T/10 (supplementary details are available from the authors on request), and supported the PD diagnosis. Based on Cp values from real-time RT-PCR one fish from each site was selected for genome sequencing and virus isolation in cell culture. The same field samples were used for generating the genome sequences and to attempt virus isolation in cell culture. As sequenced viral strains were generated from individual fish, variability observed represent intra-host variation.

Natural infection of SAV generates numerous virus variants with internally deleted genomes

At present, few SAV full-length genome sequences are available and most of these virus strains have been cultured in cells prior to sequencing. To avoid genetic changes due to cell-culture adaptation, virus genome sequencing was performed directly from clinical samples of heart tissue preserved in RNAlater. The strategy for sequencing was amplification of the entire viral genome in six overlapping PCR products. To bypass difficulties with direct sequencing of the PCR products, all PCR products were cloned and at least three clones from each PCR product were sequenced. Analysis of sequence data revealed the presence of genome deletions in many subsets of clones sequenced from each PCR product. The deletions varied in length from 1 to almost 400 nt. A closer study of the original gel electrophoresis photos of the initial PCR products revealed that deletion variants were sometimes visible as multiple bands prior to cloning. As many deletions were relatively short, some bands on gel also appeared thick, and probably consisted of two or several products of about the same size. A complete graphical presentation showing the distribution of deletions is shown in Fig. 2. The number of deletions varied between strains, ranging from only three in SAV3-9-R/10 up to 35 deletions in SAV3-8-R/10. As the majority of the deletions lead to frameshift and premature stop codons, many were assumingly encoding non-viable virus variants. The deletions were observed in all strains and in all parts of the genome, but were not distributed randomly throughout the genome but tended to be aggregated. Deletions found in nsP2 (position 2307–2366) and E1 (position 858–1006) were identical in virus strains from five and four different locations, respectively. The sequences in close proximity to the deletions were identical for the strains, and an inverted motif TGGCCCTC was found upstream and downstream of the deletion in E1. This suggests these deletions were due to sequence-dependent mechanisms, although evolution from an ancestor containing the deletion cannot be ruled out.

Genome deletions were much more common than insertions, which were found in virus strains from six

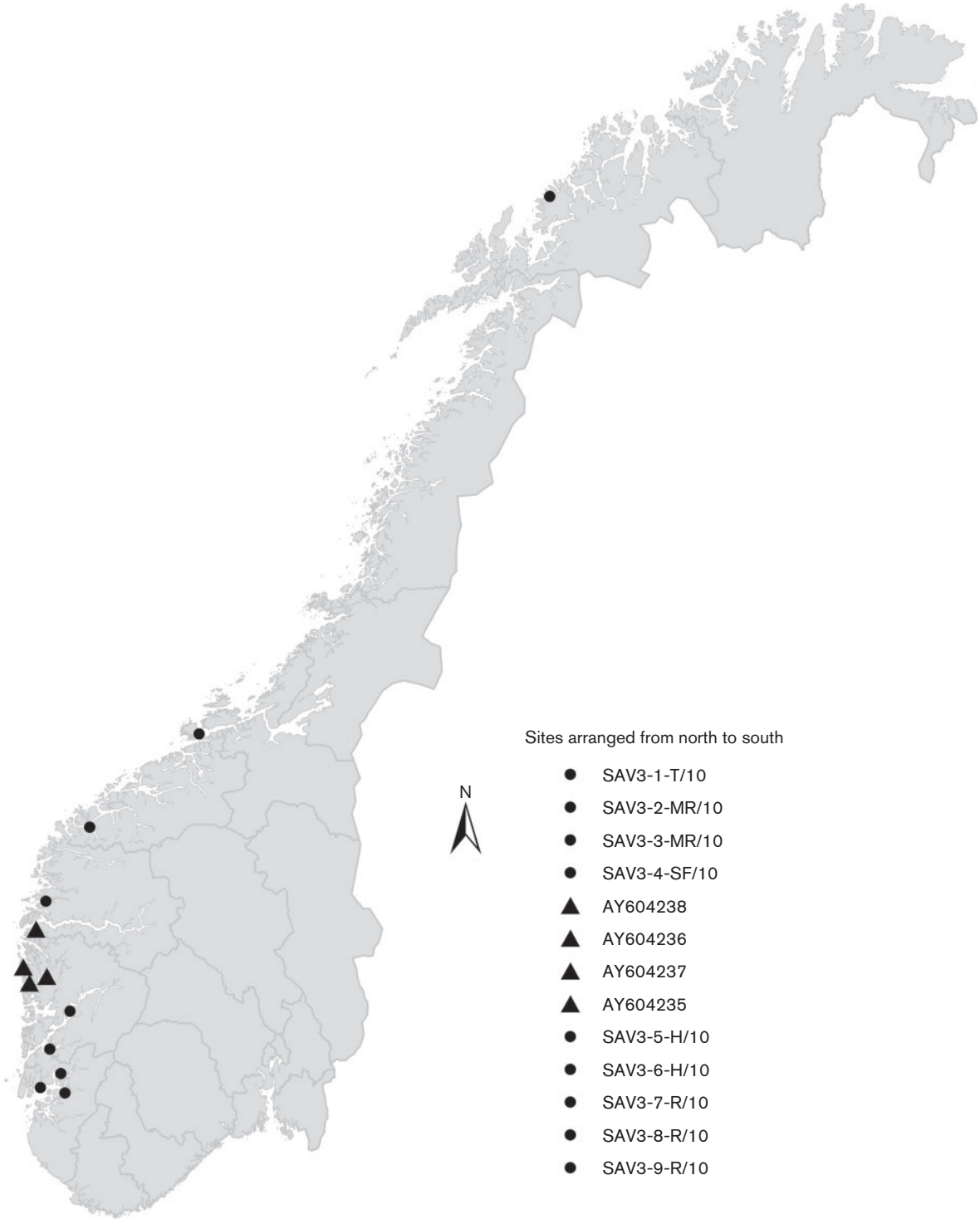

Fig. 1. Map showing the location of farms included for sequencing of SAV strains in this study. The strains are numbered from north to south along the Norwegian coastline (1–9). Capital letters denotes county of origin: T, Troms; MR, Møre og Romsdal; SF, Sogn og Fjordane; H, Hordaland; R, Rogaland. Finally, the time of sampling is given (2009/2010).

different locations. Insertions were typically of one nucleotide, except for an insertion of 4 nt found in the 3′ end of the E1 gene in one of the strains (SAV3-9-R/10). The same insertion was found in three out of four clones sequenced. The insertion interfered with the standard stop codon of E1 and resulted in an extension of 21 amino acids

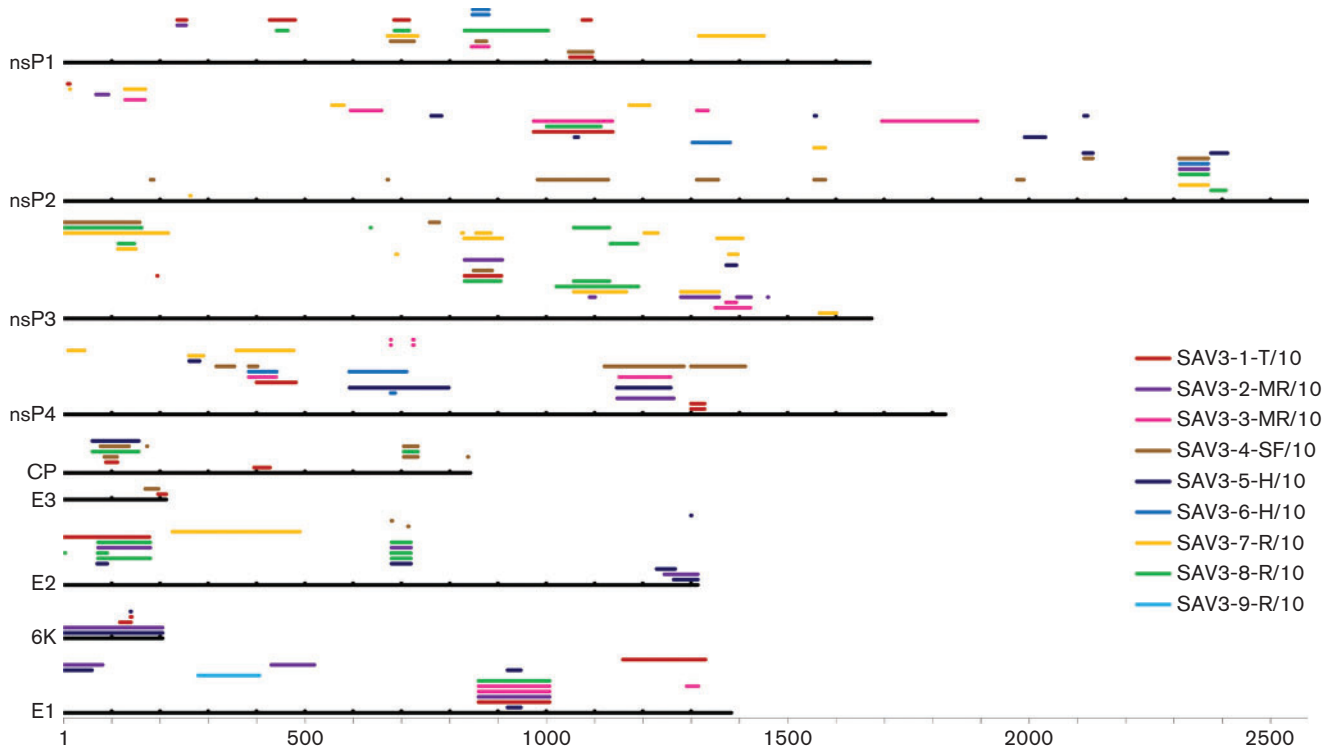

Fig. 2. Distribution of genome deletions (⩾2 nt) found in at least one clone out of 3–6 clones sequenced for each region of each virus strain. Deletion variants were found only in a subset of the clones for each population, and colour coding has been used for visualizing the distribution of deletion variants among different strains. The black lines indicate different coding regions of the contiguous genome, from 5′ to 3′, with deletions found in each region shown above each region. The consensus full-length sequences generated from a minimum of three clones per fragment did not contain deletions.

compared to the standard size of E1. Although the insertion was found in the majority of clones sequenced from this virus strain, it is not known whether this version of E1 has the ability to assemble into viral glycoprotein spikes.

Full-genome sequences showed homogeneity and were of the SAV3 subtype

The consensus sequences for full-length genomes of nine SAV strains, sampled from sites spread along the Norwegian coastline, were generated based on sequence information from a minimum of three clones per cloned fragment and did not contain deletions. Phylogenetic analysis revealed that all included virus strains were of the SAV3 subtype (results not shown), and assembled full-length sequences have been submitted to GenBank (accession numbers KC122918–KC122926). The mean nucleotide diversity among currently available full-length SAV3 sequences (nine reported in this study and four previously reported [AY604235–AY604238)], was 0.11 %, and ranged from 0.04 % to 0.21 % between the viral proteins. The corresponding numbers for each of the non-structural and structural proteins are shown in Table 1. The highest nucleotide divergence was found within the 6K gene followed by the structural protein E2, while lowest divergence was found for nsP4, nsP2 and the capsid protein.

Amino acid substitutions within available full-length consensus virus sequences, are summarized in Table 2, together with the four previously published full-length SAV3 sequences (AY604235–AY604238) which have all been collected from the same geographical region; some were based on isolates passaged on cell culture. A total of 29 amino acid positions (0.74 % of the total positions) were found to differ in at least one of the virus strains, with 22 substitutions not previously reported. However, 15 of these substitutions were infrequent and only found in one virus strain. In contrast, variability in three positions showed substitutions present in more than two strains. These include position 88 in nsP1 with an almost equal distribution of aspartic acid and valine being observed. Similarly, two positions in nsP3 showed either glutamic acid or valine in position 171, and methionine or threonine in position 517. Substitutions were also observed in 6K (residue 7), where two virus strains showed substitutions of isoleucine with threonine. This substitution has been described previously (Jansen *et al.*, 2010). Interestingly, none of the four amino acid substitutions reported to

Table 1. Comparison of mean- and maximum nucleotide diversity (%) among viral proteins of SAV3. The analyses were done using MEGA5 software, and were based on available full-length sequences: nine reported in this study and four previously reported sequences (AY604235–AY60438)

	nsP1	nsP2	nsP3	nsP4	Cp	E3	E2	6K	E1	Full
Length, nucleotides	1683	2577	1674	1827	843	213	1314	204	1383	11756
Maximum difference	0.430	0.155	0.358	0.219	0.237	0.469	0.609	0.980	0.289	0.171
Mean difference	0.131	0.055	0.168	0.042	0.052	0.132	0.180	0.214	0.111	0.108

occur *in vitro* during 20 passages of a SAV3 isolate in CHSE-214 cells were present in any of the virus strains included in this study (Karlsen *et al.*, 2006).

Intra-host genome diversity was observed as differences between sequenced clones from the same fish, and was present in all but one strain. The extent of within-fish variability varied considerably between strains, and the number of nucleotide positions showing variation ranged from none in SAV3-9-R/10 to 96 in SAV3-6-H/10. Non-synonymous substitutions within individual fish and present in more than one virus strain were found in nsP3 position 517, nsP4 position 454, E2 position 136, E2 position 220 and E1 position 454.

Phylogenetic analysis of Norwegian full-length SAV3-strains

Due to the high degree of similarity among the studied SAV3 sequences, analysis was based on full-length nucleotide sequence data for optimal resolution. The phylogenetic tree displays two clusters with acceptable bootstrap values (Fig. 3). The four northernmost virus strains formed a separate cluster with a bootstrap value of 92 %, while two previously deposited sequences (AY604237 and AY604238) formed a second cluster with a bootstrap value of 97 %. However, a geographical pattern was not present when limiting the analyses to a 451nt sequence in the E2 and including all available sequence information in GenBank (results not shown).

Primary virus isolation was achieved for only two out of eight SAV3 strains

Even though SAV can be grown in cell culture, primary virus isolation from clinically infected material can be difficult (Rowley *et al.*, 1998). Virus isolation was attempted for each of eight strains, as organ material was conserved in RNAlater only from one site and could thus not be examined by cultivation in cell culture. Heart tissue homogenate from the one or two individuals from each site showing highest viral load measured by real-time PCR was selected for cell-culture propagation, also including the individuals used for generating the full-genome sequences. Tissue homogenate was inoculated on three different cell lines (CHSE-214, CHH-1 and AGK (Munang'andu *et al.*, 2012). Primary virus isolation in CHSE-214 cells was successfully achieved from one of the eight tested strains (SAV3-9-R/10). The cytopathic effect (CPE) appeared in the third passage, with cells exhibiting CPE characteristics such as shrinkage and vacuole formation. With subsequent passages, CPE occurred earlier. The presence of SAV was confirmed by real-time RT-PCR while tests for infectious pancreas necrosis virus (IPNV) infection were negative.

CHH-1 and AGK cells were used in addition to CHSE cells, testing different variables such as volume of inoculum and culture plate formats (results not shown). Despite repeated attempts, no CPE was observed in AGK cells, while primary virus isolation was successfully managed in one more isolate (SAV3-2-MR/10) using CHH-1 cells. Here CPE was present from passage 2, and resulted in complete lysis of the cell population. This isolate was passaged four times, resulting in total cell lysis in every passage. Presence of SAV in the cell-culture medium was confirmed by real-time RT-PCR from supernatant after the second passage, while tests for IPNV infection were negative. Real-time PCR indicated no virus replication in cultures without CPE.

Defective viruses present in clinically infected heart tissue were maintained following serial passage in CHH-1 cells

Non-viable virus variants present in infected fish tissues will likely interfere with successful primary virus isolation of SAV (Roux *et al.*, 1991). As we were able to recover two of the virus strains in cell culture, we went on to study whether defective viruses containing internal genome deletions were maintained following serial passage in cells. Tissue homogenate supernatant from one of the culturable virus strains (SAV3-2-MR/10) were passaged six times in CHH-1 cells, and partially sequenced after each passage (from passage 2). The experiment was performed twice using slightly different protocols for the initial inoculum (Table 3), giving similar results. For practical reasons the analysis was limited to 2.3 kb at the 3′ end of the SAV3 genome (including 650 nt of E2, 6K and E1 gene), and CPE was present in both experiments from passage 2, resulting in complete cell lysis. Deletions were found in all (five out of five) clones amplified directly from infected fish (Table 3 and Fig. 2). After passage in cell culture, less than 20 % of the sequenced clones contained deletions. Even though virus variants with internally deleted genomes were reduced after serial passage in CHH-1 cells, there was no clear trend with regard to passage number and the frequency of sequences carrying deletions. However, it

Table 2. Amino acid diversity in studied SAV3 consensus strains compared to previously reported sequences (AY60435–AY60438). Single entries where two amino acids are given represent an equal mixture of two amino acids when an even number of clones were sequenced. An insertion of four nucleotides interfering with the standard stop codon of E1 was found in three out of four clones sequenced from SAV3-9-R/10 and resulted in an extension of 21 amino acids compared to the standard size of E1

Isolates	nsP1 66	nsP1 88	nsP1 183	nsP1 262	nsP1 292	nsP2 34	nsP2 372	nsP3 171	nsP3 350	nsP3 361	nsP3 488	nsP3 517	nsP3 545	nsP4 77	nsP4 140	nsP4 203	nsP4 248	C 117	E3 39	E2 4	E2 136	E2 206	E2 220	E2 396	6K 7	6K 33	E1 237	E1 322	E1 454	E1 461–481
AY604235/36/37/38	I	D	D	T	C	V	I/T	E/K	G	V	G	M	V	V	V	K	L	N	E/K	A/T	T	S/P	A	A/T	I	V/L	V	C	K	
SAV3-1-T/10	I	V	D	T	C	V	I	V	G	V	S	M/T	V	V	V	K	L	N	E	A	T	S	A	A	I	V	V	C	K	
SAV3-2-MR/10	I	V	D	T	C	V	I	V	G	V	S	T	V	V	V	K	L	N	E	A	T/A	S	A/V	A	T	V	V	F	K	
SAV3-3-MR/09	I	V	D	T	C	V	I	V	G	V	G	M	V	V	V	K	L	N	E	A	T	S	A	A	T	V	L	C	K	
SAV3-4-SF/10	I	V	D	T	R	V	I	V	G	V	G	T/A	V	V	V	K	L	N	E	A	T	S	A	A	I	V	V	C	K	
SAV3-5-H/10	V	D	D	A	C	A	I	E	G	V	G	M	V	V	V	K	L	N	E	A	T	S	A	A	I	V	V	C	K	
SAV3-6-H/10	I	D	D	T	C	V	I	E	G	V	G	M	A	V	V	M	V	D	E	A	T	S	A	A	I	V	V	C	K/N	
SAV3-7-R/09	I	D	D	T	C	V	I	E	G	V	G	M	V	A	I	K	L	N	E	A	T	S	A	A	I	V	V	C	R	
SAV3-8-R/10	I	D	D	T	C	V	I	E	R	A	G	M	V	V	V	K	L	N	E	A	A	S	V	A	I	V	V	C	K	
SAV3-9-R/10	I	D	N	T	C	V	T	E	G	V	G	M	V	V	V	K	L	N	E	A	T	S	A	A	I	V	V	C	K	YLEFRYINCSLG-AHPNPQGVG

remained evident that defective viruses carrying deletions were passaged together with the fully functional genomes over the first six passages. Defective viruses with the characteristic 149 nt deletion in E1 as described above were present in two clones sequenced directly from infected tissue homogenate from which strain SAV3-2-MR/10 originated. This viral deletion variant was maintained during cell passage in CHH-1 cells and found after both parallel virus passages (present after both passages 3 and 4).

DISCUSSION

This study provides the first comprehensive full-length genome comparison of nine salmonid alphavirus (SAV) strains geographically spread along the Norwegian coastline. All sequenced virus strains in this study were SAV3, the predominant subtype found in Norway at the time of sampling (Farmed fish health report 2012; www.vetinst.no). The virus genomes were sequenced directly from heart tissues, as it is well known that during passage in cell culture, adaptation can occur and genetic changes can be introduced into the virus genome (Karlsen *et al.*, 2006). Due to technical difficulties with direct sequencing, all PCR products were cloned prior to sequencing. This approach allowed us to generate legible sequences from the population of sequences carrying multiple deletions.

An important finding of our study was the presence of defective viruses carrying numerous indel mutations throughout the genome of SAV. With a total of 3–35 deletions within individual clones from the same virus strain, ranging in size from 1 to 400 nt, deletions were a predominant finding over genome insertions. The presence of larger genome deletions cannot be excluded, as sequence data were based on genome fragments of approximately 2300 nt. Deletion mutants have previously been identified in many RNA virus families both from field samples and *in vitro* (Kumaria *et al.*, 2011; Pesko *et al.*, 2012; Tsai *et al.*, 2007), and have also been described in alphaviruses (Forrester *et al.*, 2011). Virus variants containing deletions in the 6K region have previously been reported in salmonid alphavirus (SAV-1/SPDV) but were believed to be cell-culture artefacts (Weston *et al.*, 2002). The first SAV-1 sequence published represented a deletion mutant lacking 108 nt in the 6K region (Weston *et al.*, 1999), and was later corrected (Weston *et al.*, 2002). In contrast, our data provide evidence that deletions are present throughout the genome of SAV3 and that they are not caused by cell-culture artefacts. We also observed deletions in the 6K region, but these were not more prevalent than in other parts of the genome.

Viral mutants containing deletions or insertions emerge from aberrant or imprecise homologous recombination (Lai, 1992), likely produced through a copy choice mechanism (Lazzarini *et al.*, 1981). Copy choice recombination occurs when two or more viruses infect the same host cell, and the RNA polymerase dissociates from the

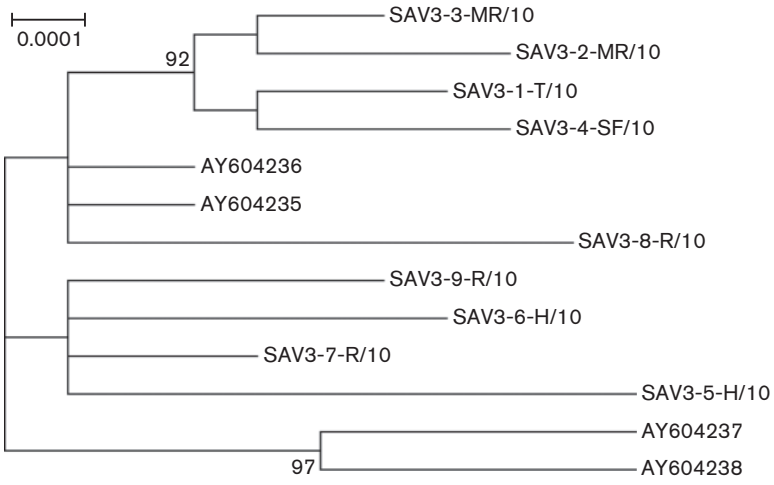

Fig. 3. A maximum-likelihood phylogenetic tree constructed from the nine full-length SAV3 sequences generated in this study and four full-length SAV3 sequences previously published (AY60435–AY60438).

template strand and switches to a homologous template at a different genomic position during synthesis, while the nascent string remains (Lai, 1992). Distribution of the observed deletions presented graphically in Fig. 2 clearly shows that the deletion variants were not randomly distributed throughout the genome, but typically clustered in certain areas. This is in conformity with publications reporting that factors such as secondary RNA structure, sequence identity and the kinetics of transcription influence template switching (Baird *et al.*, 2006; Simon-Loriere & Holmes, 2011). The presence of an inverted sequence motif immediately upstream and downstream of a deletion variant frequently found in E1 further strengthens the importance of sequence-dependent mechanisms for generation of these deletion mutants. It is not, however, possible to ensure that identical deletion variants found in several strains and collected from different locations have arisen from independent deletion events, as it is possible that these deletion variants occurred through a single ancestral deletion with subsequent spread in the viral population.

Although nucleotide insertion and deletion (indel) events, together with substitutions, represent the major mutational processes of gene evolution (Söding & Lupas, 2003), it is theorized that deletions are generated as a by-product of replication and are of no advantage to the viral population (Forrester *et al.*, 2011). Defective viruses containing only some portion of the infectious virus genome might, however, also be defined as defective interfering particles (DIPs) because they can interfere with replication of non-defective genomes through competition for cellular resources (Roux *et al.*, 1991; Stauffer Thompson *et al.*, 2009). DIPs have also been related to persistent infections in both flavivirus and alphavirus (La *et al.*, 1996; Poidinger *et al.*, 1991; Tsai *et al.*, 2007; Weiss *et al.*, 1983), and a role in maintaining persistent infections *in vivo* has been proposed (Noppornpanth *et al.*, 2007). As the SAV genome seems highly prone to internal deletions giving rise to defective viruses, the role of DIPs for persistence of PD should be further investigated.

Primary isolation of SAV poses a significant challenge (Graham *et al.*, 2008; López-Dóriga *et al.*, 2001; Rowley *et al.*, 1998), likely due to several factors including both low viral load and presence of virus neutralizing antibodies in tissues (Graham *et al.*, 2010). Even though viral RNA is present in tissues over a 9 month period indicating a carrier state, growth of SAV in cells detected by immunostaining was only successful in the acute phase (Graham *et al.*, 2010). The present work is in conformity with these observations as we were only able to recover two out of eight field strains in any of the three cell lines tested, and it is tempting to speculate that defective viral genomes being abundantly present in tissue homogenates might explain

Table 3. The occurrence of virus variants with internally deleted genomes after serial passage in CHH-1 cells. Analysis was limited to fragment 6 covering 2.3 kb at the 3′ end of the SAV3 genome. The table shows the number of clones carrying deletions as a fraction of the total number sequenced. The experiment was performed twice using high volume of inoculum during the first two passages in experiment 1

Experiment	Homogenate	Passage					
		1	2	3	4	5	6
1	5/5	–	3/6	0/6	1/6	0/6	1/6
2	5/5	–	0/6	1/3	2/6	0/4	–

difficulties related to primary virus isolation. Despite the confirmed presence of viral RNA by PCR in infected tissues prior to isolation in cell culture, we observed that a large number of these viral genomes were incomplete, being unable to sustain an infection by themselves. These defective viral variants will also be potent activators of the interferon-induced responses, and it is well known that alpha interferon as part of the immune response is a strong inhibitor of SAV3 replication *in vitro* (Xu *et al.*, 2010). DIPs cannot sustain infection themselves but are efficiently replicated and encapsidated using proteins produced by wild-type viral genomes (García-Arriaza *et al.*, 2004), which was also the case for SAV. When SAV was successfully recovered in cell culture, defective viral genomes were maintained after six passages in CHH-1 cells.

It has been speculated that only minor populations of viral quasispecies present in clinical SAV samples have possibilities of growth in cell cultures (Weston *et al.*, 2002), and a serine to proline substitution at position 206 in the E2 protein has been associated with the appearance of CPE in CHSE-214 cells (Karlsen *et al.*, 2006). It was also suggested to affect *in vivo* virulence although this has not been documented experimentally. In contrast, Fringuelli *et al.* (2008) suggested that other factors were responsible for *in vivo* virulence after finding proline in all E2 sequences generated. Our study confirmed that proline at position 206 in the E2 protein is not a prerequisite for viral propagation in cell culture as the SAV3-2-MR/10 isolate successfully recovered in cells revealed serine at position 206 both prior to and after serial passage in cells.

Our data suggest that recombination events occur frequently during replication of salmonid alphavirus, leading to viral mutants containing deletions and insertions. Recombination appears more frequently in positive-sense ssRNA viruses, than in negative sense ssRNA viruses (Simon-Loriere & Holmes, 2011), and is well known to occur in alphaviruses (Schlesinger & Weiss, 1994). Evidence for homologous recombination in alphaviruses was confirmed in sequence analysis of Western equine encephalitis virus (WEEV) that appears to have arisen by homologous double crossovers during natural infection with Sindbis virus and Eastern equine encephalitis virus (EEEV) (Hahn *et al.*, 1988). Recombination might play a role in future vaccine strategies, and the use of alphaviruses as viral vectors carrying homologous and heterologous genes are novel strategies to protect against intracellular pathogens (Bråve *et al.*, 2007; Smerdou & Liljeström, 1999). Despite the fact that replication-competent viral vectors are highly effective there are legitimate concerns about their safety. Replication-deficient vectors with a helper vector system are able to compensate for this imperfection (Smerdou & Liljeström, 1999), although frequent recombination events in salmonid alphavirus will have safety implications for such vaccines.

The present results are in agreement with previous reports stating a homogeneous genetics for SAV3 in Norway, but reports for the first time that numerous viral deletion mutants, in all parts of the genome, are generated during natural infections with SAV. We suggest recombination as an explanation for generation of these virus variants. Defective viruses, missing parts of their genome and present in the infected tissues, provide a possible explanation for difficulties regarding primary virus isolation. Novel DNA sequencing techniques providing in-depth viral genome sequence data should shed light on the variety of mutants present in SAV populations and the dynamics of these during infection processes. Future work should address whether the defective viral genomes behave as DIPs or are merely a by-product of replication, and explore their role in primary virus isolation and in sustaining persistent infections of SAV.

METHODS

Sampling and background information. Farmed Atlantic salmon (*Salmo salar* L.) were sampled during the 2009–2010 grow-out season from nine different seawater sites spread along the Norwegian coastline. The geographical distribution of the sites included in this study is shown in Fig. 1. The farms were chosen based on prior PD diagnosis. From each site, 10–20 fish were killed by a sharp blow to the head prior to necropsy. Heart specimens were collected and parallel samples preserved on RNAlater (Invitrogen) and transport medium containing Leibovitz's L-15 (Invitrogen) supplemented with 100 μg gentamicin ml^{-1} (Sigma Aldrich). Parallel samples from heart, pancreas and skeletal muscle were submerged in 10 % phosphate-buffered formalin and processed for paraffin-embedment and sectioning according to standard methods. Tissue specimens were stained with haematoxylin and eosin using standard methods. From one of the sites however, only samples preserved in RNAlater were available.

RNA isolation. Total RNA was extracted from heart tissue of individual fish using the RNeasy Fibrous tissue mini kit (Qiagen), according to the kit protocol. The tissue was homogenized in RLT buffer with β-mercaptoethanol using steel beads in a mixer mill MM301 (Retsch) for 2 min at 20 Hz. RNA was quantified using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). From cell-culture supernatants RNA was purified using QIAamp Viral RNA Mini kit (Qiagen) including optional on-column DNase digestion according to the manufacturer's specifications.

cDNA synthesis and real-time PCR. cDNA synthesis was performed using the Transcriptor High Fidelity cDNA Synthesis kit (Roche Applied Science), which includes proofreading activity. In general, 1–4 μg RNA was denatured at 65 °C for 10 min, and cDNA synthesis was carried out at 50 °C for 30 min using a combination of random hexamers and oligo (dT) primers. Initially, all fish were screened for presence of SAV by real-time PCR analysis using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) in a LightCycler 480 Real-time PCR system (Roche Applied Science). In short, 2 μl undiluted cDNA was used as template in a 20 μl reaction with primers SAV forward (5′-CAGTGAAATTCGATAAGAAGTGCAA) and SAV reverse (5′-TGGGAGTCGCTGGTAAAGGT). The primer annealing temperature was 60 °C and the real-time PCR was run for 40 cycles according to the manufacturer's recommendations.

Amplification of viral genome, cloning and sequencing. PCR amplification of the full viral genome was performed using Advantage 2 Polymerase Mix (Clontech). For practical reasons amplification of the entire genome was performed in six fragments, each overlapping

the next fragment by approximately 150 nt. The primer sets were designed using Vector NTI advance 11.0 software (Invitrogen) and defined products between 2000 and 2300 base pairs. Primer sequences are shown in Table S1 (available in JGV Online). PCR was performed using 2 µl undiluted cDNA as template at standard conditions: 95 °C for 1 min, 30 cycles at 95 °C for 10 s, 60–68 °C for 1 min and final extension at 68 °C for 3 min. The resulting products were visualized in a 1 % agarose gel with SYBR Safe staining (Invitrogen). The products were excised from the gel and purified with Qiagen gel extraction kit (Qiagen). PCR products were ligated into the pCR 2.1 vector using TOPO TA cloning kit (Invitrogen) before subsequently being transformed into competent OneShot TOP10 bacterial cells (Invitrogen). The insert of purified plasmids of a minimum of three clones from each fragment was sequenced using commercial services (Eurofins MWG operon or GATC biotech) using both standard vector primers and internal forward and reverse primers designed for each fragment.

Virus propagation in cell culture. From each site heart tissue samples with the highest virus load shown by real-time PCR were selected for cell-culture propagation. Heart tissues were homogenized in Leibovitz's L-15 medium (Invitrogen) supplemented with 50 µg gentamicin ml^{-1} (Sigma Aldrich), 1/10 (w/v). After centrifugation, supernatant was further diluted 1/10 in Leibovitz's L-15 medium containing 2 % FBS (Sigma Aldrich) and 50 ng ml^{-1} gentamicin before being inoculated on Chinook salmon embryo cells (CHSE-214). Extensive optimizations with various combinations of amount inoculate and culture plate formats were performed. Two other cell lines were also inoculated in parallel: Asian Grouper Kidney (AGK) and Chum salmon heart (CHH-1) cells. All cells were grown at 15 °C up to 21 days post-inoculation, frozen and thawed, and cell-culture supernatant were harvested after centrifugation. Supernatants were passaged 2–6 times in all cell lines. Supernatant from non-CPE cultures were also analysed by real-time PCR for SAV infection after 10–14 days. Cultures showing CPE were tested for both IPNV and SAV. IPNV testing was performed using the same real-time PCR method as described earlier, with forward primer GACTGGAG-GTAAAAGGCATCGA and reverse CCGAACTCCGACATGGTGTT.

Serial passage of isolate SAV3-2-MR/10. Isolate SAV3-2-MR/10 was serial passaged in CHH-1 cell line in two parallel experiments (Table 3). In experiment 1, cells in a 24-well plate were initially inoculated with 250 µl original homogenate per well in the first passage, 250 µl supernatant transferred to new wells in the second and then 100 µl supernatant was used as inoculum from the third to the sixth passage. In experiment 2, 5 µl inoculum was used per well in all passages. Cell-culture supernatant was harvested after 7–9 days when total cell lysis had occurred. SAV3 genome sequence analysis was performed on the supernatants after each passage as described earlier. The analysis was limited to fragment 6 covering 2.3 kb at the 3′ end of the SAV3 genome.

Bioinformatics. Consensus full-length sequences for the nine virus strains, all from different sites, were aligned in Contig Express of Vector NTI advance 11.0 software. The consensus sequences were generated based on sequence information from a minimum of three clones per fragment and consensus was determined on the basis of simple majority. The full-length nucleotide sequences from the nine fish farms were imported into the MEGA5 software for further analysis. In addition four previously published full-length SAV3 sequences were downloaded from GenBank. The sequences were aligned using the MUSCLE (Edgar, 2004) algorithm within MEGA5. To find the optimal substitution model the statistical program R (R Development Core Team, 2010) was used together with the 'ape' library (Paradis *et al.*, 2004). In addition, the PhyML program (Guindon *et al.*, 2009) was run in R to carry out maximum-likelihood estimation. The model that obtained the lowest AIC (Aikaike Information Criterion) was chosen. According to the AIC a maximum-likelihood phylogenetic tree with 1000 bootstrap replications was constructed using Hasegawa–Kishino–Yano model with invariant sites. Bootstrap values of 60 and above was included in the output. The tree was constructed from 11756 nt from nsP1 to E1.

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REFERENCES

Baird, H. A., Galetto, R., Gao, Y., Simon-Loriere, E., Abreha, M., Archer, J., Fan, J., Robertson, D. L., Arts, E. J. & Negroni, M. (2006). Sequence determinants of breakpoint location during HIV-1 intersubtype recombination. *Nucleic Acids Res* **34**, 5203–5216.

Bråve, A., Ljungberg, K., Wahren, B. & Liu, M. A. (2007). Vaccine delivery methods using viral vectors. *Mol Pharm* **4**, 18–32.

Castric, J., Baudin-Laurencin, F., Brèmont, M., Le Ven, A. & Bèarzotti, M. (1997). Isolation of the virus responsible for sleeping-disease in experimentally infected rainbow-trout *Oncorhynchus Mykiss*. *Bull Eur Assoc Fish Pathol* **17**, 27–30.

Durden, L. A., Linthicum, K. J. & Turell, M. J. (1992). Mechanical transmission of Venezuelan equine encephalomyelitis virus by hematophagous mites (Acari). *J Med Entomol* **29**, 118–121.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792–1797.

Forrester, N. L., Guerbois, M., Adams, A. P., Liang, X. & Weaver, S. C. (2011). Analysis of intrahost variation in Venezuelan equine encephalitis virus reveals repeated deletions in the 6-kilodalton protein gene. *J Virol* **85**, 8709–8717.

Fringuelli, E., Rowley, H. M., Wilson, J. C., Hunter, R., Rodger, H. & Graham, D. A. (2008). Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. *J Fish Dis* **31**, 811–823.

García-Arriaza, J., Manrubia, S. C., Toja, M., Domingo, E. & Escarmís, C. (2004). Evolutionary transition toward defective RNAs that are infectious by complementation. *J Virol* **78**, 11678–11685.

Graham, D. A., Wilson, C., Jewhurst, H. & Rowley, H. (2008). Cultural characteristics of salmonid alphaviruses–influence of cell line and temperature. *J Fish Dis* **31**, 859–868.

Graham, D. A., Fringuelli, E., Wilson, C., Rowley, H. M., Brown, A., Rodger, H., McLoughlin, M. F., McManus, C., Casey, E. & other authors (2010). Prospective longitudinal studies of salmonid alphavirus infections on two Atlantic salmon farms in Ireland; evidence for viral persistence. *J Fish Dis* **33**, 123–135.

Graham, D. A., Frost, P., McLaughlin, K., Rowley, H. M., Gabestad, I., Gordon, A. & McLoughlin, M. F. (2011). A comparative study of marine salmonid alphavirus subtypes 1-6 using an experimental cohabitation challenge model. *J Fish Dis* **34**, 273–286.

Graham, D. A., Fringuelli, E., Rowley, H. M., Cockerill, D., Cox, D. I., Turnbull, T., Rodger, H., Morris, D. & Mc Loughlin, M. F. (2012). Geographical distribution of salmonid alphavirus subtypes in marine farmed Atlantic salmon, Salmo salar L., in Scotland and Ireland. *J Fish Dis* **35**, 755–765.

Griffin, D. E. (2007). Alphaviruses. In *Virology*, 5th edn, pp. 1023–1067. Edited by M. Knipe & M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Guindon, S., Delsuc, F., Dufayard, J. F. & Gascuel, O. (2009). Estimating maximum likelihood phylogenies with PhyML. *Methods Mol Biol* **537**, 113–137.

Hahn, C. S., Lustig, S., Strauss, E. G. & Strauss, J. H. (1988). Western equine encephalitis virus is a recombinant virus. *Proc Natl Acad Sci U S A* **85**, 5997–6001.

Hjortaas, M. J., Skjelstad, H. R., Taksdal, T., Olsen, A. B., Johansen, R., Bang-Jensen, B., Ørpetveit, I. & Sindre, H. (2013). The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, *Salmo salar* L., in Norway. *J Fish Dis* **36**, 71–74.

Hodneland, K., Bratland, A., Christie, K. E., Endresen, C. & 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.

Jansen, M. D., Gjerset, B., Modahl, I. & Bohlin, J. (2010). Molecular epidemiology of salmonid alphavirus (SAV) subtype 3 in Norway. *Virol J* **7**, 188.

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

Kumaria, R., Iyer, L. R., Hibberd, M. L., Simões, E. A. & Sugrue, R. J. (2011). Whole genome characterization of non-tissue culture adapted HRSV strains in severely infected children. *Virol J* **8**, 372.

Lai, M. M. (1992). RNA recombination in animal and plant viruses. *Microbiol Rev* **56**, 61–79.

Lazzarini, R. A., Keene, J. D. & Schubert, M. (1981). The origins of defective interfering particles of the negative-strand RNA viruses. *Cell* **26**, 145–154.

Linn, M. L., Aaskov, J. G. & Suhrbier, A. (1996). Antibody-dependent enhancement and persistence in macrophages of an arbovirus associated with arthritis. *J Virol* **77**, 407–411.

López-Dóriga, M. V., Smail, D. A., Smith, R. J., Doménech, A., Castric, J., Smith, P. D. & Ellis, A. E. (2001). Isolation of salmon pancreas disease virus (SPDV) in cell culture and its ability to protect against infection by the 'wild-type' agent. *Fish Shellfish Immunol* **11**, 505–522.

McLoughlin, M. F. & Graham, D. A. (2007). Alphavirus infections in salmonids–a review. *J Fish Dis* **30**, 511–531.

McLoughlin, M. F., Nelson, R. T., Rowley, H. M., Cox, D. I. & Grant, A. N. (1996). Experimental pancreas disease in Atlantic salmon Salmo salar post-smolts induced by salmon pancreas disease virus (SPDV). *Dis Aquat Organ* 26, 117–124.

McLoughlin, M. F., Graham, D. A., Norris, A., Matthews, D., Foyle, L., Rowley, H. M., Jewhurst, H., MacPhee, J. & Todd, D. (2006). Virological, serological and histopathological evaluation of fish strain susceptibility to experimental infection with salmonid alphavirus. *Dis Aquat Organ* **72**, 125–133.

McVicar, A. H. (1987). Pancreas disease of farmed Atlantic Salmon, *Salmo salar,* in Scotland: epidemiology and early pathology. *Aquaculture* **67**, 71–78.

Munang'andu, H. M., Fredriksen, B. N., Mutoloki, S., Brudeseth, B., Kuo, T. Y., Marjara, I. S., Dalmo, R. A. & Evensen, O. (2012). Comparison of vaccine efficacy for different antigen delivery systems for infectious pancreatic necrosis virus vaccines in Atlantic salmon (Salmo salar L.) in a cohabitation challenge model. *Vaccine* **30**, 4007–4016.

Nelson, R. T., McLoughlin, M. F., Rowley, H. M., Platten, M. A. & Mccormick, J. I. (1995). Isolation of a toga-like virus from farmed Atlantic salmon *Salmo salar* with pancreas disease. *Dis Aquat Organ* **22**, 25–32.

Noppornpanth, S., Smits, S. L., Lien, T. X., Poovorawan, Y., Osterhaus, A. D. & Haagmans, B. L. (2007). Characterization of hepatitis C virus deletion mutants circulating in chronically infected patients. *J Virol* **81**, 12496–12503.

Paradis, E., Claude, J. & Strimmer, K. (2004). APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics* **20**, 289–290.

Pesko, K. N., Fitzpatrick, K. A., Ryan, E. M., Shi, P. Y., Zhang, B., Lennon, N. J., Newman, R. M., Henn, M. R. & Ebel, G. D. (2012). Internally deleted WNV genomes isolated from exotic birds in New Mexico: function in cells, mosquitoes, and mice. *Virology* **427**, 10–17.

Petterson, E., Sandberg, M. & Santi, N. (2009). Salmonid alphavirus associated with *Lepeophtheirus salmonis* (Copepoda: Caligidae) from Atlantic salmon, *Salmo salar L. J Fish Dis* **32**, 477–479.

Poidinger, M., Coelen, R. J. & Mackenzie, J. S. (1991). Persistent infection of Vero cells by the flavivirus Murray Valley encephalitis virus. *J Gen Virol* **72**, 573–578.

R Development Core Team (2010). *R: A Language and Environment for Statistical Computing*. Vienna: Foundation for Statistical Computing.

Roux, L., Simon, A. E. & Holland, J. J. (1991). Effects of defective interfering viruses on virus replication and pathogenesis in vitro and in vivo. *Adv Virus Res* **40**, 181–211.

Rowley, H. M., Doherty, C. E., McLoughlin, M. F. & Welsh, M. D. (1998). Isolation of salmon pancreas disease virus (SPDV) from farmed Atlantic salmon, *Salmo salar* L., in Scotland. *J Fish Dis* **21**, 469–472.

Schlesinger, S. & Weiss, B. G. (1994). Recombination between Sindbis virus RNAs. *Arch Virol Suppl* **9**, 213–220.

Simon-Loriere, E. & Holmes, E. C. (2011). Why do RNA viruses recombine? *Nat Rev Microbiol* **9**, 617–626.

Smerdou, C. & Liljeström, P. (1999). Non-viral amplification systems for gene transfer: vectors based on alphaviruses. *Curr Opin Mol Ther* **1**, 244–251.

Söding, J. & Lupas, A. N. (2003). More than the sum of their parts: on the evolution of proteins from peptides. *Bioessays* **25**, 837–846.

Stauffer Thompson, K. A., Rempala, G. A. & Yin, J. (2009). Multiple-hit inhibition of infection by defective interfering particles. *J Gen Virol* **90**, 888–899.

Tsai, K. N., Tsang, S. F., Huang, C. H. & Chang, R. Y. (2007). Defective interfering RNAs of Japanese encephalitis virus found in mosquito cells and correlation with persistent infection. *Virus Res* **124**, 139–150.

Weiss, B., Levis, R. & Schlesinger, S. (1983). Evolution of virus and defective-interfering RNAs in BHK cells persistently infected with Sindbis virus. *J Virol* **48**, 676–684.

Weston, J. H., Welsh, M. D., McLoughlin, M. F. & Todd, D. (1999). Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, *Salmo salar L. Virology* **256**, 188–195.

Weston, J., Villoing, S., Brémont, M., Castric, J., Pfeffer, M., Jewhurst, V., McLoughlin, M., Rødseth, O., Christie, K. E. & other authors (2002). Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. *J Virol* **76**, 6155–6163.

Weston, J. H., Graham, D. A., Branson, E., Rowley, H. M., Walker, I. W., Jewhurst, V. A., Jewhurst, H. L. & Todd, D. (2005). Nucleotide sequence variation in salmonid alphaviruses from outbreaks of salmon pancreas disease and sleeping disease. *Dis Aquat Organ* **66**, 105–111.

Xu, C., Guo, T. C., Mutoloki, S., Haugland, Ø., Marjara, I. S. & Evensen, Ø. (2010). Alpha interferon and not gamma interferon inhibits salmonid alphavirus subtype 3 replication in vitro. *J Virol* **84**, 8903–8912.

Xu, C., Guo, T. C., Mutoloki, S., Haugland, Ø. & Evensen, Ø. (2012). Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon. *Vet Res* **43**, 78.

III

Experimental piscine alphavirus RNA recombination *in vivo* yields both viable virus and defective viral RNA

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Abstract

RNA recombination in non-segmented RNA viruses is important for viral evolution and documented for several virus species through *in vitro* studies. During viral RNA synthesis, the RNA-dependent RNA polymerase switches template but holds on to the nascent RNA strand. Here we confirm viral RNA recombination *in vivo* using an alphavirus, the SAV3 subtype of Salmon pancreas disease virus. The virus causes pancreas disease in Atlantic salmon and subsequently heavy losses in European salmonid aquaculture. Atlantic salmon were injected with a SAV3 6K-gene deleted cDNA plasmid, encoding a non-viable variant of SAV3, together with a helper cDNA plasmid encoding structural proteins and 6K only. Later, SAV3-specific RNA was detected and recombination of viral RNA was confirmed. Virus was grown from plasmid-injected fish and shown to infect and cause pathology in salmon. Subsequent cloning of PCR products confirming recombination, documented imprecise homologous recombination creating RNA deletion variants in fish injected with cDNA plasmid, corresponding with deletion variants previously found in SAV3 from field. This is the first experimental documentation of alphavirus RNA recombination in an animal model and provides new insight into the production of defective virus RNA.

Introduction

Alphavirus is one of two genera in the family *Togaviridae* [1]. They are small, enveloped viruses that infect a broad range of insect and vertebrate hosts. Currently there are 31 assigned virus species within the genus (International Committee on Taxonomy of Viruses, 2014), where two virus species infecting marine animals are identified, southern elephant seal virus (SESV) and salmon pancreas disease virus (SPDV) [2,3]. Alphaviruses are classified as arboviruses (arthropod-borne virus) transmitted to vertebrates by hematophagous insects [1]. SPDV infects salmonids and can be transmitted without an insect vector [4]. SPDV, also called salmonid alphavirus (SAV), is divided into subtype 1-6 [5] and causes pancreas disease (PD) and severe disease problems with high economic losses in salmonid aquaculture in Europe. The SPDV genome is an 11.9kb, positive sense ssRNA molecule, capped (5´) and polyadenylated (3´) and serves directly as mRNA for translation of the viral replicase when it enters a host cell [1,6]. As for all alphavirus, the RNA molecule contains two open reading frames (ORFs). The first ORF encodes a polyprotein which is processed into four non-structural proteins (nsP1-4), responsible for transcription and replication of viral RNA. The second ORF is transcribed as a subgenomic mRNA and translated into a polyprotein subsequently processed into the structural proteins (capsid, E3, E2, 6K and E1). The cleavage sites in both polyproteins are deduced from amino acid sequence homologies with other alphaviruses [3,6-8]. E2 and E1 are the envelope glycoproteins and it is shown for other alphaviruses that they are associated as a heterodimer that migrates together with the membrane-associated 6K protein to the plasma membrane [9].

Recombination in RNA viruses refers to the process that occurs within a single genomic segment during virus replication. The polymerase switches templates during RNA synthesis, a so-called copy choice model [10,11]. Two types of RNA recombinations have been defined, homologous and heterologous, both documented in alphavirus *in vitro* [12]. The first known incidence of alphavirus recombination is the emergence of Western equine encephalitis virus (WEEV), a recombination of an Eastern equine encephalitis (EEEV)-like virus and a Sindbis-like virus [13], later giving rise to Highlands J

virus (HJV), and Fort Morgan virus (FMV) [1]. Recombination of Sindbis virus RNA *in vitro* has been documented experimentally in transfected cells in the early nineties [12,14]. RNA recombination may also result in formation of defective interfering (DI) RNAs [15]. Thus RNA virus recombination plays an important role in virus variability and evolution, and can also play a role in rescuing viral genomes by repairing mutation errors introduced during RNA replication [16]. Experimentally, RNA recombination of alphaviruses has not been shown *in vivo* across all susceptible species.

In a previous study on the SAV3 subtype of SPDV, we documented that infectious virus (with full-length genome) is present alongside with numerous RNA deletion mutants in heart of clinically diseased Atlantic salmon, and we hypothesized that recombination of genomes was the explanation for this [17]. Using reverse genetics, we have shown that infectious virus can be rescued from cell lines transfected with full length SAV3 (FL-SAV3) plasmid [18]. When cell cultures was transfected with a full length but 6K-deleted construct (FL-Δ6K), viral proteins were expressed in transfected cells, but viable, infectious virus was not formed [18]. When we co-transfected cell cultures with the FL-Δ6K plasmid and a helper plasmid encoding the structural proteins including 6K (Helper-6K), infectivity was rescued through viral RNA recombination *in vitro* and formation of infectious virus [18]. This provided us with a tool to explore if the same recombination would occur *in vivo*. We used Atlantic salmon, one of the species susceptible to SAV3 infection, and we have documented for the first time recombination of alphavirus RNA in an animal model. Virus was grown in cell culture from heart of FL-Δ6K/Helper-6K injected fish and shown to be pathogenic to salmon in experimental challenge.

Results

Presence of viral RNA transcripts in plasmid-injected fish

First, we aimed to document presence of viral RNA transcripts following injection with plasmid/plasmid mixtures. Atlantic salmon parr were injected intramuscularly (i.m.) with a combination of a cDNA plasmid expressing full-length SAV3 genome with the 6K-gene deleted (FL-

Δ6K) and a helper cDNA vector (Helper-6K) encoding the structural genes including 6K (Fig. 1). SAV3-specific RNA, part of the E2 gene, was detected by real-time PCR in heart and head kidney in 3 of 10 individuals at 3 weeks post injection (wpi, Fig. 2a). Thereafter, 1 of 6 individuals was positive for viral RNA at 4 wpi and 3 of 10 fish at 5 wpi, in total 7 PCR-positive fish (Fig. 2a). The amount of viral RNA detected (given as Cp-values) varied between 21.8 and 31.6, with no obvious trends (increase/decrease) over time post injection. Viral RNA was not detectable by PCR at 1 and 2 wpi (not shown). No SAV3-specific RNA was found in any organ at any time point post injection in the group injected with FL-Δ6K alone or in negative control fish (not shown). Negative control fish were also negative at start of the experiment.

Experimental infection with live virus results in pathological changes in heart, pancreas and skeletal muscle when given at doses above 10^3 $TCID_{50}$ [19]. Here, no lesions were recorded (by histological examination) in any of the sampled organs. To ascertain that PCR positive signals did not originate from injected plasmids, all RNA was DNase-treated at time of isolation and absences of plasmid DNA contamination was also verified by real-time PCR on RNA from selected samples without transcription into cDNA (preceding PCR). These findings indicate transcription of viral genome and/or replication of virus, and the fact that RNA was not detected by PCR in samples during the first two weeks post injection corroborates the same.

Recombination is confirmed *in vivo*

The 6K gene is required for replication and production of infectious virus [18]. To verify presence of 6K in the 7 PCR-positive fish (by real-time PCR, Fig. 2a), we examined these fish using conventional PCR and primers yielding an amplicon covering the 6K gene (380bp) and all were found positive (Fig. 2a), indicating that a replicating virus including 6K gene in the genome could have resulted from recombination. To confirm this, we amplified a larger product, 2.9 kb spanning the end of nsp4 and the structural proteins including 6K into E1 (Fig. 3a). The primers were designed to include a unique 67nt stretch of nsp4 for the SAV3-FL-Δ6K plasmid while the entire 6K frame was unique for the

Helper-6K (Fig. 3a). A product including both sequences would only be present if recombination had occurred. In all of the 7 individuals positive for the 380bp 6K amplicon, the 2.9kb amplicon was produced to detectable levels. Cloning and sequencing confirmed that sequences, unique for the two plasmids, were included (Fig. 2a), with the exception of fish 3w-7, which resulted in a 2.9kb amplicon with too low concentration for cloning and sequencing (Fig. 2a). Collectively, these results confirm recombination *in vivo*.

Primary isolation of recombinant SAV3 in CHH-1 cell culture

Our next focus was to show that virus could be isolated from target/internal organs. Organ material (heart/kidney) from the real-time PCR positive fish (Fig. 2a) were processed for inoculation onto permissive cells, CHH-1, with subsequent passaging. No cytopathic effect was evident during the first two passages of any tissue samples tested, but we obtained positive immunofluorescent staining for SAV3 spike proteins of 2nd passage cells inoculated with heart tissue from individuals 3w-1 and 3w-2, 3 days post inoculation (dpi) (Fig. 2b). At 3rd passage, cytopathic effect (CPE) appeared for fish no. 3w-1 and 3w-2 (Fig. 2b). For the latter, CPE was also found for kidney tissue inoculated cells. Early signs of CPE appeared, as vacuoles at 6 dpi and later at 10 dpi swelling and detached single cells were dominant. Subsequent passages showed typical CPE at 4 dpi, progressing to cell lysis at 8-10 dpi (Fig. 2b). Supernatant from 5th passage of heart tissue, fish 3w-2 (isolate 3w-2H), was used for challenge studies. The obtained titer was $10^{6.8}$ $TCID_{50}$/ml, and before subsequent use RNA including recombined sequences were confirmed as described above (amplification of unique SAV3 FL-Δ6K encoded RNA and Helper-6K RNA). The entire viral genome of 3w-2H isolate was also sequenced confirming it to be identical to the original SAV3-H10 genome sequence, with the exception of a C1375G mutation of nsp3, resulting in a Q459E substitution.

Recombinant SAV3 induces pathology in Atlantic salmon parr

Since we were not able to confirm any pathological changes in the plasmid-injected fish we used isolate 3w-2H (5th passage, Fig. 2b) to challenge Atlantic salmon parr by i.m. injection in a second *in*

vivo experiment. SAV3 RNA was detected by PCR in all individuals sampled at 4 and 6 wpc (Fig. 2c). Pathological changes specific for SAV3-infection were found in heart, 4/6 fish at 4 wpi and 6/6 at 6 wpi, and in exocrine pancreas in 6/6 fish at both time points (Fig. 2c). Fish with typical changes in pancreas [20] (Supplementary Fig. 1a) also showed presence of SAV3 antigens, shown by immunohistochemistry (Supplementary Fig. 1b).

Imprecise homologous recombination creates RNA deletion variants

One thing that puzzled us was the observation that several of the plasmid injected fish at week 4 and 5 were PCR positive for the 2.9kb amplicon documenting recombination (Fig. 2a) while at the same time, we had difficulties with growing any virus from internal organs (fish 4w-5, 5w-4, 5w7, and 5w-10; Fig. 2b). Detailed analyses of 4-6 clones from each fish show that recombinations occurred and were homologous resulting in the complete 2.9kb. However, imprecise (homologous) recombinations generated either during initial rounds of transcription or during virus replication resulting in deletions (Fig. 3b), also occurred. The deletions varied from one nt deleted to more than 2.2kb missing fragment, and varied in position and number per clone sequenced. Noteworthy, some of the deletions were identical or varied by only a few nucleotides at start and end positions in several clones (Fig. 3b), and interestingly deletion positions were similar to what has previously been reported by us from field outbreaks [17]. None of the clones had deletions similar to the injected FL-Δ6K plasmid (i.e. only 6K gene deleted). In addition all clones contained single synonymous and nonsynonymous substitutions, some also resulting in stop codons, evenly spread in the 2.9kb region.

RNA deletion variants occur also in virus injected fish

We cloned and sequenced 5 clones targeting the 2.9kb amplicon from the 5th passage supernatant of virus isolate 3w-2H that was used for challenge (Fig. 2c). No deletions or single nucleotide substitutions were found in any of the clones. Then, and similar to what was done to confirm recombination in the plasmid-injected fish, RNA isolated from heart of 3w-2H challenged fish (Fig 2c) were cloned and sequenced (2.9 kb amplicon) and deletions reappeared. 3 to 5 amplicon clones

from 3 individuals sampled at 4wpi were sequenced. Of the 13 clones, deletions were found in 4 (Supplementary Fig. 2) and one clone had nucleotide substitution in two adjacent nt's, resulting in one amino acid change. This shows that SAV3 accumulates mutations/deletions in experimental *in vivo* challenges at early time post infection.

Deletions are frequently initiated at loops of unpaired nts

In an attempt to shed light on mechanisms possibly involved in generation of deletions, we used Mfold web server [21] to predict the RNA structure in some regions of the genome where deletions frequently occur. Deletions in or including the full 6K gene can be explained by a polymerase jump across loops of unpaired nts or from one loop to a loop in close vicinity. At these positions, the polymerase meets stems of helices of base paired nts, which might cause the polymerase release from the template (Fig. 4a). In E2, deletions are found putatively resulting from similar polymerase release, but here it jumps to another loop of unpaired nts located more distantly (Fig. 4b).

Discussion

Here we present the first documentation of experimental alphavirus RNA recombination in an animal model. An infectious and virulent virus originated from two i.m. injected cDNA plasmid vectors, each unable to generate infectious progeny alone. Recombination occurred *in vivo* in Atlantic salmon and the resulting virus was shown to be infectious in cell culture. The isolated virus was infectious and resulted in specific pathology in the target organs, *i.e.* heart and pancreas. The recombination is shown to be imprecise as several viral RNA deletion mutants were found after plasmid injection. The deletions were of varying size and genome positions but the location in viral genome was identical or similar for several sequenced clones, and some positions were identical to deletions previously seen in SAV3 RNA in field infections in salmon [17].

RNA Recombination as an evolutionary force in alphavirus is already documented when WEEV and the WEEV antigenic complex descended from a recombination event between a Sindbis-like virus and

EEEV [1,13]. Thus, recombination seems to be a founding feature of alphaviruses. Still, it has previously not been documented to occur in experimental studies *in vivo* for any Alphavirus species, including marine Alphaviruses. At a general level, studies of RNA virus recombination show that the RNA secondary structure might play a role. This was suggested from the observation that the RNA-dependent RNA polymerases pauses at regions of strong secondary structure resulting in release of the RNA polymerase and the nascent strand from the template [22-24]. Some regions of the SAV3 genome aggregate deletions, as shown here where deletions in and covering 6K gene are highly frequent, also supported by previous reports for variant SAV subtypes [3,6,17]. Mfold secondary structure modeling is indicative of deletions in the 6K gene and can relate to a polymerase jump across loops of unpaired nts, *i.e.* deleting stems of helices of base paired nts. Similarly, examples of deletions in the E2 gene can be explained by a polymerase release at a loop of unpaired nts (upstream of a basepaired helix stem) with a "jump" to another loop of unpaired nts. In general, all deletions analyzed on the two secondary structure predictions was related to a "jump" either within or between such loops. Interesting to note is that amplicon sequences from fish tissue revealed a deletion pattern partly similar to what we published from fish samples from field outbreaks [17]. Deletions found in the present study, after injection of plasmids or live virus, are also represented in data from 2013, although the size of the deletions could vary with a few nts. In total, the aggregation of deletions in certain "hot spots" of the viral genome is confirmed and secondary structures can be one of the underlying mechanisms involved. Putative "hot spots" in the SAV3 genome represent relatively small deletions where many result in frameshift downstream of the deletions resulting in non-viable viruses.

Some regions of the genome are more prone to be deleted than others while the size, position and frequency in each amplicon varied. In fact, when the 2.9kb amplicon was produced to confirm recombination, a "ladder" of several shorter products were often seen by gel electrophoresis (Supplementary Fig. 4). These shorter products were not sequenced, but it is reasonable to believe that they represent specific amplicons either resulting from a large deletion or multiple smaller

deletions resulting in a short amplicon. Similarly, large deletions in the 2.9kb amplicon region resulting in shorter amplicons, were seen previously when viral RNA recombination was studied *in vitro* [18]. Further, amplicons with large deletions came up from sequencing when approximately 2.9kb amplicons were cut out from the gel, seemingly as contaminants of smaller PCR amplicons. This could be explained by the PCR enriching shorter amplicons present at high concentration relatively to the 2.9kb amplicons and a low separation resolution on the gel runs, resulting in low precision. Generation of large deletions fits with what was discussed above where the RNA polymerase pause and "jump" at regions of strong secondary structure. Still, "secondary" deletions in an already deleted RNA copy may result in the deleted fragment increasing in length with repeated rounds of replication.

Alphavirus RNA recombination have earlier been confirmed in experimental *in vitro* studies using RNA transcripts [12,14]. Deletion variants of similar characteristics as found here, and suggested to be caused by RNA recombination, has been shown in other alphavirus both *in vivo* and *in vitro* with no cDNA variants of the viral genomes were present [25]. Also, equal deletions as presented here were found in SAV3 RNA under field conditions [17]. In total, this supports that the recombination shown in the present work is related to RNA and not the injected DNA plasmids.

Unlike other alphaviruses, SAV is transferred without a vector, which makes the potential host range larger. SAV variants do not only infect salmonids, and while the wild reservoir of SAV remains elusive, SAV subtype 5 have been found in several wild caught fish species in the Shetland Islands and north-east coast of Scotland [26]. Transfer through a vector might restrict genetic variation (bottlenecks) and single amino acids mutations have been associated with overcoming host range barriers especially at the level of the vector [27,28]. At the same time as spreading through an insect vector might restrict genetic variation of the virus, the vector serves as a virus pool. For SAV a complicating factor is that Atlantic salmon is a migrating species, which will provide the virus with a paucity of hosts available for infection (under natural conditions in the wild). Thus, it is tempting to speculate that high

mutation/recombination rate can provide a genetic plasticity that will benefit the virus in terms of cross-species transmission, also discussed for other Alphaviruses like Chikungunya virus and Venezuelan equine encephalitis virus (VEEV) [27]. A recent study has shown that different variants of SAV isolated from farmed fish in Norway, Scotland and Ireland, represent separate introductions from wild fish species, yet to be identified [29]. The referred study show that introduction to farmed salmon species very likely occurred as separate events in areas of close proximity. These findings might argue for a viral genome of high plasticity and it has been speculated that the ancestral alphavirus originates from the marine environment [30]. To achieve a transfer to terrestrial animal species a viral ancestor with a wide host specter and high genomic plasticity would be beneficial.

Against the ideas above stands a theory that deletions are generated as a by-product of replication and are of no advantage to the viral population. Also, that recombination in RNA viruses is not a result of natural selection that in itself creates an advantageous genotype but rather an effect of a low-fidelity polymerase and high replication rates producing occasional beneficial combinations [25,31,32]. Recombination is also linked to the production of defective interfering particles (with similar truncated viral genomes as seen here) which propagates and accumulates at high MOIs and attenuates the virus [14,24,33]. Our findings do not corroborate this. Although we were not able to sequence more than one clone of the 2.9kb amplicon from the fish 3w-2 tissue, the gel electrophoresis after PCR show a ladder of smaller amplicons representing sequences with one or several deleted regions of large size (Supplementary Fig. 3). Sequencing of the five PCR clones of the 2.9kb amplicon after the 3w-2H isolate was passaged five times in cell culture show no deletions, no laddering was observed on the gel. We previously found that while deletion mutants are present at high concentrations during natural infections, passage in cell culture tends to result in decrease of deleted RNAs [17]. This would indicate a purifying selection with passage rather than formation of accumulating DI variants. DI particles are well known among Alphaviruses and similar deletion mutants as shown here in the 6K gene are also described previously as non-interfering in VEEV [25].

255 An interesting observation here is the low viral load shown by real-time PCR in the 3w-2 fish from
256 which virus was grown. In contrast we did not succeed in growing any virus from e.g. heart tissue of
257 fish 4w-5 where the real-time PCR indicated approximately 1000 times higher concentration of viral
258 RNA (Fig. 2a and b). These finding corroborate previous studies where primary isolation of SAV from
259 diseased/infected fish has been challenging [17,34-36]. For many years the understanding has been that
260 SAV3 in Norway isolated from farmed salmon is genetically homogenous with little sequence
261 divergence or variability, which is confirmed when comparing sequences of cultivable virus isolates.
262 The observed difficulties isolating virus from tissue might be due to a high concentration of defective
263 RNA, which could indicate that presence of deletion mutants is of some relevance to replication and
264 packaging/release of the virus. More studies dedicated to the prevalence, characteristics and impact
265 of these deletions are needed.

266 In summary, we have confirmed viral RNA recombination *in vivo,* using the SAV3 subtype of SPDV.
267 This is the first experimental documentation of *in vivo* recombination in *Alphavirus* genus resulting in
268 an infectious virus.
269

Material and methods

Virus and cells

The study was performed using genomic sequences for salmonid alphavirus subtype 3 (SAV3) H10 isolate (GenBank ref. no. JQ799139) [37], a subtype of salmonid pancreas disease virus (SPDV).

Chum salmon (*Oncorhynchus keta*) heart cells (CHH-1) were used to grow the virus and were obtained from the European Collection of Cell Cultures (ECACC), maintained at 20°C in Leibovitz`s L-15 media with GlutaMAX™ (Invitrogen), supplemented with 5% fetal bovine serum (FBS) (Sigma Aldrich) and 50 µg/ml gentamicin.

Model animal

Atlantic salmon parr (*Salmo sala*r L.) for both experimental *in vivo* studies were obtained from the Norwegian institute for water research (NIVA), Solbergstrand, Norway. The average individual weight was 50g in the first and 28g in second *in viv*o study. Both studies were conducted in fresh water (city water) at the aquarium facilities of The Norwegian University of Life Science/Norwegian Veterinary Institute in Oslo, Norway. In the first *in vivo* study, the water temperature was 12±1°C, while in the second study the water temperature was 7°C the first two weeks and 10±1°C the remaining four weeks. Six randomly selected fish used in the first study, were tested for persistent infection with infectious pancreatic necrosis virus (IPNV) by PCR and low levels of viral RNA was detected in two. Fish were negative for salmon pancreas disease virus (n=10). Fish were anesthetized with 30-40mg/liter benzocain prior to handling, *i.e.* injection or sampling. Fish were euthanized by a sharp blow to the head after being put in deep anesthesia. All experiments were approved by The Norwegian Animal Research Authority and the local IACUC of the Norwegian University of Life Sciences and carried out in compliance with national regulation for the use of animals in experimental research.

295 **SAV3 6K-deleted cDNA plasmid and helper cDNA plasmid**

296 Construction of the SAV3 6K-deleted cDNA plasmid (FL-Δ6K) and the helper cDNA plasmid (Helper-
297 6K) are described in Guo *et al* 2014. In brief, a full length infectious clone of SAV3 cDNA was inserted
298 into the pTurboFP635-N vector (Evrogen). Between the vector's CMV promoter and the 5'UTR region
299 of the full-length genome cDNA, a T7 promoter and a hammerhead (HH) self-cleavage ribozyme
300 sequence was included. The plasmid was designated pSAV3-FL and used subsequently as a template
301 for generation of SAV3 6K-deleted cDNA and helper cDNA plasmids. The 6K gene was deleted by PCR
302 using primers defining the deletion, followed by circularization of the amplified product, resulting in
303 FL-Δ6K plasmid. The SAV3 helper 6K cDNA plasmid was constructed by deleting the replicon genes
304 nsP1, nsP2, nsP3 and almost entire nsP4 from the pSAV3-FL, resulting in 100 nt at nsP4 3'-end and
305 intact internal untranslated regions and genes encoding the structural proteins plus 6K, termed
306 Helper-6K (Fig. 1). The plasmids were copied in and purified from transformed competent OneShot
307 TOP10 bacterial cells (Invitrogen), using QIAGEN®Plasmid Maxi kit.

308 **SAV3 FL-Δ6K cDNA plasmid and helper-6K cDNA vector injected in Atlantic salmon parr**

309 110 Atlantic salmon parr were included in the study. 50 individuals were randomly selected for
310 injection with a plasmid mix of FL-Δ6K and Helper-6K and 50 were selected for injection with FL-Δ6K
311 only. The fish were injected intramuscularly with 20 µg of each plasmid (40 µg total) diluted in dH_2O
312 in a total of 100 µl for the FL-Δ6K/Helper-6K group, or 20 µg/fish for FL-Δ6K group (100 µl injection
313 volume). Fish from the two groups were kept in separate tanks and a third tank with 10 non-injected
314 parr was included as negative control (dH_2O injected). Ten fish per experimental tank were sampled
315 once a week for five weeks post injection (wpi). At 4 wpi, the number of fish sampled from the two
316 experimental tanks was reduced to 6 and 7, due to mortality experienced between 3 and 4 wpi. The
317 mortality was caused by a *Saprolegnia* infection, commonly seen in recirculating systems and fish
318 handled/stressed. The infection was treated with malachite green (10mg/L). From the dH_2O -injected
319 fish in the control tank no mortalities occurred and five individuals were sampled at each time point,

0 weeks and 5 weeks after onset of the experiment (n=10 total).

Sampling and sample preservation and preparation

Heart and head kidney tissue were collected and preserved in RNAlater® (Invitrogen). Parallel samples of heart and head kidney were preserved on transport medium (Leibovitz`s L-15 supplemented with 100mg/ml gentamicin and 30% glycerol). Samples from heart and pancreas were submerged in 10% phosphate-buffered formalin, embedded in paraffin and stained with hematoxylin and eosin using standard methods. Histopathological changes in heart and pancreas was evaluated as described [4].

RNA isolation, transcription and PCR based methodology

RNA was extracted from heart and kidney tissue using the RNeasy® Fibrous tissue mini kit on heart specimens and RNeasy®mini kit (Qiagen) on kidney, all according to the kit protocols. On-column DNAse treatment was included for all samples. The tissue was homogenized in kit RLT buffer with β-mercaptoethanol using steel beads in a FastPrep-24 homogenizer (MP Biomedicals). RNA was extracted from cell supernatants using QIAamp Viral RNA mini kit (Qiagen) according to the protocol of the manufacturer. RNA was quantified using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA was denatured at 95°C for 2.5 minutes and transferred directly to ice immediately before cDNA synthesis was performed with SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific), according to the protocol, using 500ng RNA in the reaction for heart and kidney samples. For a few samples with RNA concentration too low to include 500ng, maximum volume of RNA possible was used. Real-time PCR analysis was conducted with Platinum SYBR Green qPCR SuperMix UDG (Thermo Fischer Scientific) as earlier described [17] using 2 µl 1:2 diluted cDNA as template in a LightCycler® 96 Real-time PCR system (Roche), with primers targeting E2 gene. A conventional PCR was also used as an alternative to real-time PCR, where Q5® High-Fidelity DNA Polymerase (New England BioLabs) was used in combination with primers E1- Fwd

and E1- Rev (Supplementary Table) defining a 225bp product, and run for 35 cycles. A PCR confirming presence of the full 6K gene was also performed using primers 6K Fwd and Insert rev (Supplementary Table) and run for 35 cycles. The resulting 225 and 380 bp product was visualized by gel electrophoresis.

PCR and sequencing of amplicons

Recombination of virus specific RNA was verified by PCR amplification of a product using a forward primer specific for sequences unique to SAV3 FL-Δ6K encoded RNA, Insert Fwd, and a reverse primer resulting in inclusion of unique sequences of Helper-6K encoded RNA, Insert Rev (Supplementary Table). The resulting product after a successful and precise recombination would be sized 2.9kb and include 67bp of nsP4 (nt 7622-7688 in genome) unique to FL-Δ6K in the 5' end and full 6K gene unique to Helper-6K at the 3' end, but products of any size, which still including parts of the unique sequences would also indicate recombination. The PCR was run using DyNAzyme EXT DNA Polymerase (Thermo Scientific™) or Q5® High-Fidelity DNA Polymerase for 35 cycles. The products were visualized by gel electrophoresis and products of approximately 2.9kb were excised and purified with Qiagen gel extraction kit (Qiagen). PCR products were ligated into the pCR 2.1 vector using TOPO TA cloning kit (Invitrogen) before subsequently being transformed into competent OneShot TOP10 bacterial cells (Invitrogen), after manufacturer's procedures. The insert of purified plasmids of one to six clones was sequenced by commercial services (GATC biotech) using standard vector primers and sequencing primers (Supplementary Table). Full genome sequencing was performed by PCR amplification using four primer sets (Supplementary table) defining overlapping fragments covering the complete genome excluding 5' and 3' end sequences and the amplified PCR products (around 3 kb for each fragment) were purified and sequenced (GATC biotech) without subsequent cloning.

Isolation of recombinant virus from fish tissue

Tissues were homogenized in L-15 medium supplemented with 100 µg/mL gentamicin, centrifuged and obtained supernatant was diluted 1:10 in L-15 (2% FBS and 50 µg/ml gentamicin). IPNV was neutralized by adding K262, a custom made rabbit serum to IPNV [38] to the tissue homogenate, and incubated for one hour at room temperature. Heart tissue supernatant (from homogenate) was further diluted 1:10 and 1:100, and kidney 1:50 and 1:100. 24 well plates with 50% confluent CHH-1 cells was inoculated with 250 µl per well of the different dilutions. The inoculated cells were propagated at 15°C until the 3rd passage with the following passages at 10°C. At passage, virus supernatant was harvested by one freeze-thaw cycle (passage 1-3) at 14dpi unless cytopathic effect (CPE) was observed (late passages). Supernatant was clarified by centrifugation before transferring to a naïve cell culture. Anti-IPNV antiserum (K262) was added to the inoculum up to 4th passage, and real-time PCR confirmed all supernatants IPNV negative. IPNV real-time PCR was performed as described above using specific IPNV primers (Supplementary table).

SAV3 titer of the 5th and final supernatant were determined by endpoint dilution on CHH-1 cells grown in 96-well plates, estimating the $TCID_{50}$ by the method of Kärber [39]. Detection of SAV3 viral proteins in cultured CHH-1 cells was performed by IF assay on the 2nd passage using a polyclonal antibody against SAV3 structural proteins [18].

Recombinant SAV3 *in vivo* challenge

A challenge study was conducted using 22 Atlantic salmon parr. In the experimental tank, 12 fish were injected intramuscularly with 100µl $10^{6.8}$ $TCID_{50}$ of recombined SAV3 (3w-2H isolate) from the 5th cell culture passage. 10 salmon parr were injected with 100 µl PBS, kept in a separate tank and used as negative controls. Six fish were sampled from the challenge tank at 4 and 6 wpi. Five fish from the negative control tank were sampled at 0 and 6 wpi. Sampling was performed and the samples preserved and analyzed as described above. Presence of SAV3 antigens in situ in heart and pancreas was documented by immunohistochemistry [20].

References

1 Strauss, J. H. The alphaviruses: gene expression, replication, and evolution. *Microbiol.Rev.* **58**, 491-562 (1994).

2 La, L. M. *et al.* Arbovirus of marine mammals: a new alphavirus isolated from the elephant seal louse, *Lepidophthirus macrorhini*. *J.Virol.* **75**, 4103-4109 (2001).

3 Weston, J. H., Welsh, M. D., McLoughlin, M. F. & Todd, D. Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, *Salmo salar L*. *Virology* **256**, 188-195 (1999).

4 Xu, C., Guo, T. C., Mutoloki, S., Haugland, O. & Evensen, O. Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon. *Vet.Res.* **78**, 1-43 (2012).

5 Fringuelli, E. *et al.* Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. *J.Fish Dis.* **31**, 811-823 (2008).

6 Weston, J. *et al.* Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. *J.Virol.* **76**, 6155-6163 (2002).

7 Villoing, S., Bearzotti, M., Chilmonczyk, S., Castric, J. & Bremont, M. Rainbow trout sleeping disease virus is an atypical alphavirus. *J.Virol.* **74**, 173-183 (2000).

8 Welsh, M. *et al.* Biochemical characterization of salmon pancreas disease virus. *J.Gen.Virol.* **81**, 813-820 (2000).

9 Loewy, A., Smyth, J., von Bonsdorff, C. H., Liljestrom, P. & Schlesinger, M. J. The 6-kilodalton membrane protein of Semliki Forest virus is involved in the budding process. *J.Virol.* **69**, 469-475 (1995).

10 Copper, P. D., Steiner-Pryor, A., Scotti, P. D. & Delong, D. On the nature of poliovirus genetic recombinants. *J.Gen.Virol.* **23**, 41-49 (1974).

11 Kirkegaard, K. & Baltimore, D. The mechanism of RNA recombination in poliovirus. *Cell* **47**, 433-443 (1986).

12 Raju, R., Subramaniam, S. V. & Hajjou, M. Genesis of Sindbis virus by in vivo recombination of nonreplicative RNA precursors. *J.Virol.* **69**, 7391-7401 (1995).

13 Hahn, C. S., Lustig, S., Strauss, E. G. & Strauss, J. H. Western Equine Encephalitis-Virus is a recombinant virus. *Proc.Natl.Acad.Sci.U.S.A.* **85**, 5997-6001 (1988).

14 Weiss, B. G. & Schlesinger, S. Recombination between Sindbis virus RNAs. *J.Virol.* **65**, 4017-4025 (1991).

15 Lazzarini, R. A., Keene, J. D. & Schubert, M. The origins of defective interfering particles of the negative-strand RNA viruses. *Cell* **26**, 145-154 (1981).

16 Lai, M. M. RNA recombination in animal and plant viruses. *Microbiol.Rev.* **56**, 61-79 (1992).

17 Petterson, E., Stormoen, M., Evensen, O., Mikalsen, A. B. & Haugland, O. Natural infection of Atlantic salmon (*Salmo salar* L.) with salmonid alphavirus 3 generates numerous viral deletion mutants. *J.Gen.Virol.* **94**, 1945-1954 (2013).

18 Guo, T. C., Johansson, D. X., Haugland, O., Liljestrom, P. & Evensen, O. A 6K-deletion variant of Salmonid alphavirus is non-viable but can be rescued through RNA recombination. *PLoS.One.* **9**, e100184 (2014).

19 McLoughlin, M. F. & Nelson, R. T. (Google Patents, 1999).

20 Taksdal, T. *et al.* Pancreas disease in farmed Atlantic salmon, Salmo salar L., and rainbow trout, Oncorhynchus mykiss (Walbaum), in Norway. *J.Fish.Dis* **30**, 545-558 (2007).

21 Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nuc.Acids Res.* **31**, 3406-3415 (2003).

22 Nagy, P. D. & Bujarski, J. J. Targeting the site of RNA-RNA recombination in brome mosaic virus with antisense sequences. *Proc.Natl.Acad.Sci.U.S.A.* **90**, 6390-6394 (1993).

23 Havelda, Z., Dalmay, T. & Burgyan, J. Secondary structure-dependent evolution of Cymbidium ringspot virus defective interfering RNA. *J.Gen.Virol* **78** 1227-1234 (1997).

24 Pathak, K. B. & Nagy, P. D. Defective Interfering RNAs: Foes of Viruses and Friends of Virologists. *Viruses* **1**, 895-919 (2009).
25 Forrester, N. L., Guerbois, M., Adams, A. P., Liang, X. & Weaver, S. C. Analysis of intrahost variation in Venezuelan equine encephalitis virus reveals repeated deletions in the 6-kilodalton protein gene. *J.Virol.* **85**, 8709-8717 (2011).
26 Snow, M. *et al.* Detection of salmonid alphavirus RNA in wild marine fish: implications for the origins of salmon pancreas disease in aquaculture. *Dis.Aquat.Organ.* **91**, 177-188 (2010).
27 Allison, A. B., Stallknecht, D. E. & Holmes, E. C. Evolutionary genetics and vector adaptation of recombinant viruses of the western equine encephalitis antigenic complex provides new insights into alphavirus diversity and host switching. *Virology* **474**, 154-162 (2015).
28 Myles, K. M., Kelly, C. L., Ledermann, J. P. & Powers, A. M. Effects of an opal termination codon preceding the nsP4 gene sequence in the O'Nyong-Nyong virus genome on Anopheles gambiae infectivity. *J.Virol.* **80**, 4992-4997 (2006).
29 Karlsen, M., Gjerset, B., Hansen, T. & Rambaut, A. Multiple introductions of salmonid alphavirus from a wild reservoir have caused independent and self-sustainable epizootics in aquaculture. *J.Gen.Virol.* **95**, 52-59 (2014).
30 Forrester, N. L. *et al.* Genome-scale phylogeny of the alphavirus genus suggests a marine origin. *J.Virol.* **86**, 2729-2738 (2012).
31 Simon-Loriere, E. & Holmes, E. C. Why do RNA viruses recombine? *Nat.Rev.Microbiol.* **9**, 617-626 (2011).
32 Worobey, M. & Holmes, E. C. Evolutionary aspects of recombination in RNA viruses. *J.Gen.Virol.* **80** 2535-2543, doi:. (1999).
33 Poirier, E. Z. *et al.* Low-fidelity polymerases of alphaviruses recombine at higher rates to overproduce defective interfering particles. *J.Virol.* (2015).
34 Graham, D. A., Wilson, C., Jewhurst, H. & Rowley, H. Cultural characteristics of salmonid alphaviruses-influence of cell line and temperature. *J.Fish Dis.* **31**, 859-868 (2008).
35 Lopez-Doriga, M. V. *et al.* Isolation of salmon pancreas disease virus (SPDV) in cell culture and its ability to protect against infection by the 'wild-type' agent. *Fish Shellfish Immunol.* **11**, 505-522 (2001).
36 McLoughlin, M. F. & Graham, D. A. Alphavirus infections in salmonids-a review. *J.Fish.Dis.* **30**, 511-531 (2007).
37 Xu, C. *et al.* Alpha interferon and not gamma interferon inhibits salmonid alphavirus subtype 3 replication *in vitro*. *J.Virol.* **84**, 8903-8912 (2010).
38 Evensen, O. & Rimstad, E. Immunohistochemical identification of infectious pancreatic necrosis virus in paraffin-embedded tissues of Atlantic salmon (*Salmo salar*). *J.Vet.Diagn.Invest.* **2**, 288-293 (1990).
39 Kärber, G. Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. . *Exp.Pathol.Pharmakol.*, 480-483 (1931).

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Author contributions

E.P. contributed with the idea and design of the study, performed the main experimental work and wrote the main body of the paper. T-Z.G. contributed with the plasmid constructs, primer design and performed the full genome sequencing and also contributed to the IF assay, data and design of figures, interpretation of results and commented on the manuscript. Ø.E. conceived the project and contributed to the design of the study, interpretation of results and writing of the manuscript. A.B.M contributed to the design of the study, supervision of the experimental work, interpretation of results, data and design of figures and writing of the manuscript.

Competing financial interests

The authors declare no competing financial interest.

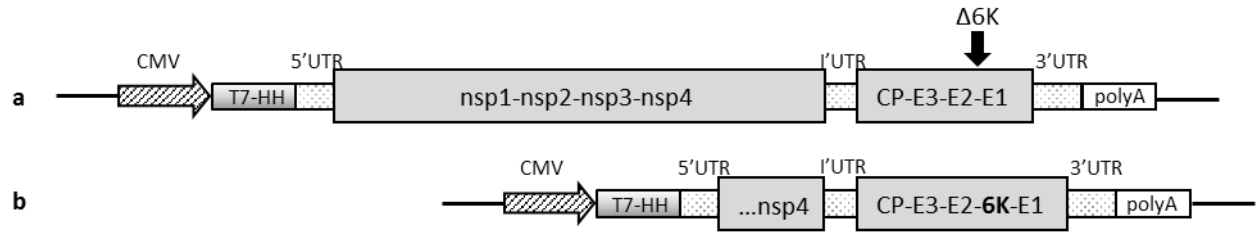
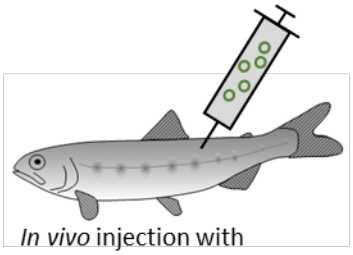


Figure 1 | Organization map of SAV3 cDNA in plasmids.

a) SAV3 6K-deleted cDNA plasmid (FL-Δ6K): the entire 6K gene was removed from SAV3 full-length genome.

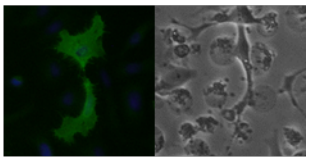
b) Helper cDNA plasmid (Helper-6K): 100 bases of nsp4 C-terminal together with the whole 26S subgenome containing 6K gene were included in the helper plasmid. HH: hammerhead ribozyme sequence, a self-cleavage ribozyme providing the exact 5' viral end after RNA transcription from cDNA plasmid.

a



In vivo injection with combined cDNA plasmids

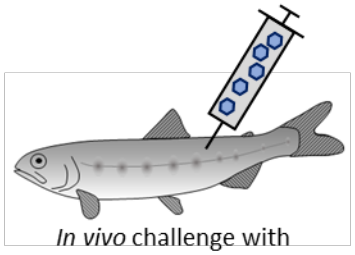
b



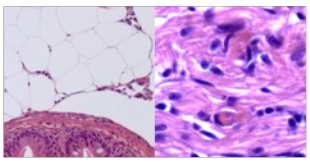
Isolation of recombined SAV3 in cell culture

Sampling	Fish #	Organ	SAV3 E2 specific real-time PCR (Cp)	Presence of 6K-gene	RNA recombination confirmed	3rd passage	5th passage
3wpi	3w-1	Heart	25.3	+	-	+	+
		Kidney	25.0	+	+	-	-
	3w-2	**Heart**	**31.6**	**+**	**-**	**+**	**+**
		Kidney	30.0	+	+	+	+
	3w-7	Heart	27.9	+	-	-	
		Kidney	31.3	+	(+)	-	
4wpi	4w-5	Heart	21.8	+	+	-	
		Kidney	27.9			-	
5wpi	5w-4	Heart	23.4	+	+	-	
		Kidney	27.7			-	
	5w-7	Heart	25.3	+	+	-	
		Kidney	30.3			-	
	5w-10	Heart	24.6	+	+	-	
		Kidney	29.9			-	

c



In vivo challenge with recombined SAV3, 3w-2H



Sampling	Fish #	SAV3 specific PCR Heart	SAV3 pathology Pancreas/Heart
6wpc	1	+	+/+
	2	+	+/+
	3	+	+/+
	4	+	+/+
	5	+	+/+
	6	+	+/+

Figure 2 | Experimental overview and main results.

a) Atlantic salmon injected with a combination of SAV3 FL-Δ6K and Helper-6K cDNA plasmids. Results show fish with presence of SAV3 RNA in heart and kidney tissue as Cp-values (individuals with no presence are not shown) at the specified sampling time (weeks post injection (wpi)) and as presence of 6K-gene found by PCR (+). The outcome of PCR analysis for RNA recombination in heart and kidney tissue is shown, and + denotes presence of an amplicon with unique sequences from SAV3-FL-Δ6K and Helper-6K plasmids, confirmed by sequencing. Four to seven plasmid clones were sequenced from each PCR-positive fish, with the exception of fish 3w-2 where a very low concentration of the 2.9kb product only resulted in one clone. All clones include the sequence unique for the SAV3-FL-Δ6K plasmid and at least one clone per fish also includes 6K or incomplete 6K sequences unique for the Helper-6K. No clones show a sequence equal to any of the two plasmids alone. Brackets indicate presence of amplicon where sequences were not obtained; – denotes no amplicon present.

b) Primary isolation of recombinant SAV3 in CHH-1 cell culture. Left image show example of positive staining for SAV3 spike proteins in 2nd passage of cells inoculated with heart tissue from fish 3w-1 and 3w-2. CPE was not seen in this passage. Right image show example of CPE, 5th passage. CPE (+) in cell cultures inoculated with tissue from the fish positive for SAV3 RNA. Results for 3rd and 5th passage shown. – denotes no CPE.

c) Atlantic salmon injected with SAV3 from the supernatant of the 5th passage (fish 3w-2, heart, isolate 3w-2H). Results show fish with presence (+) of SAV3 RNA in heart tissue and evidence of pathology characteristic of SAV3 infection in pancreas and heart by light microscopy. Image show loss of exocrine pancreas located in perivisceral fat tissue (left) and necrotic myocytes in spongiosum of the heart ventricle (right).

Results in **a** and **b** are related to isolate 3w-2H (bold), used in **c.** Grey shading indicates no analyses performed.

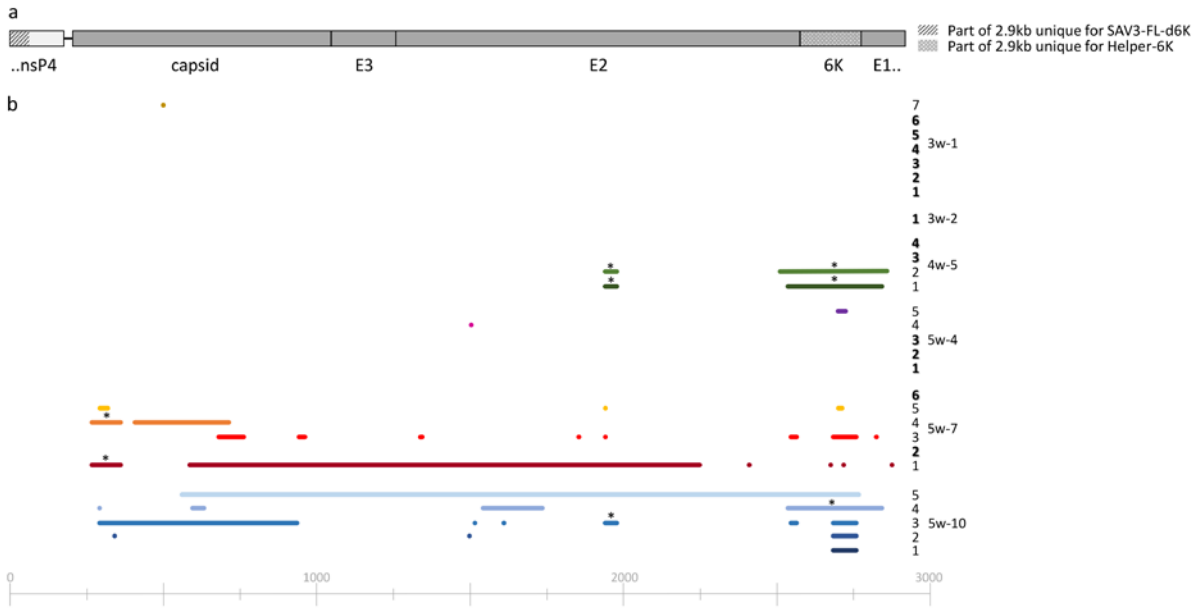
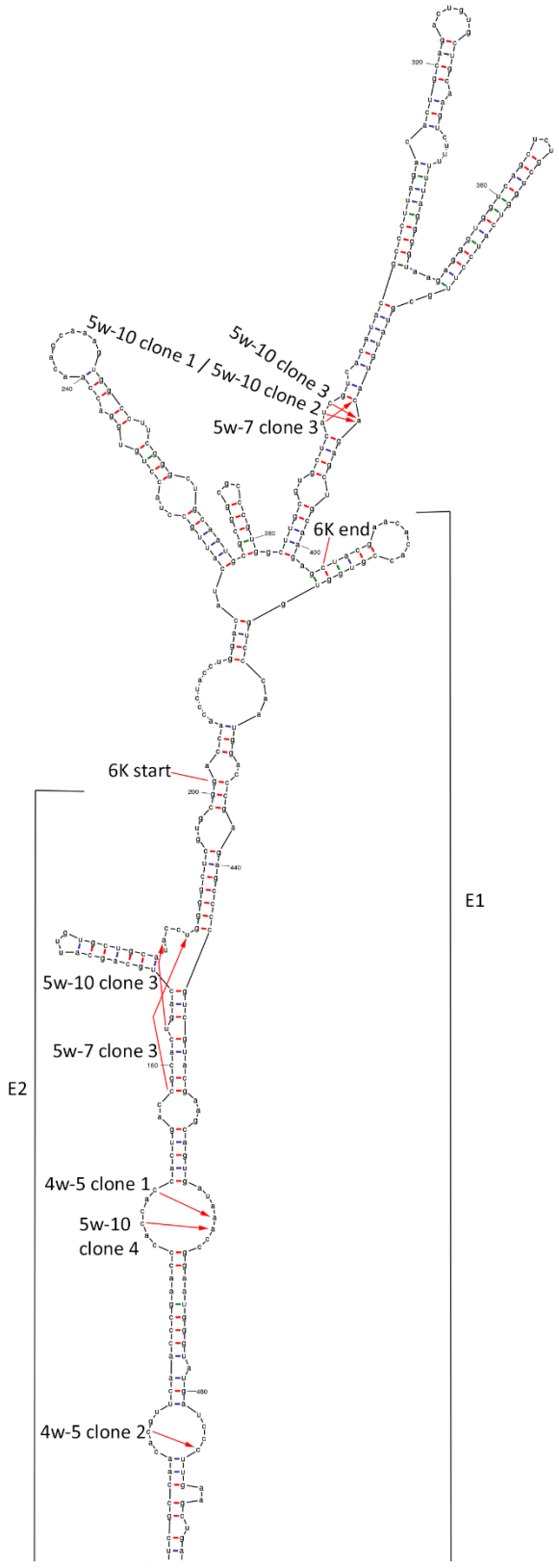


Figure 3 | Sequencing of PCR products from recombined virus RNA.

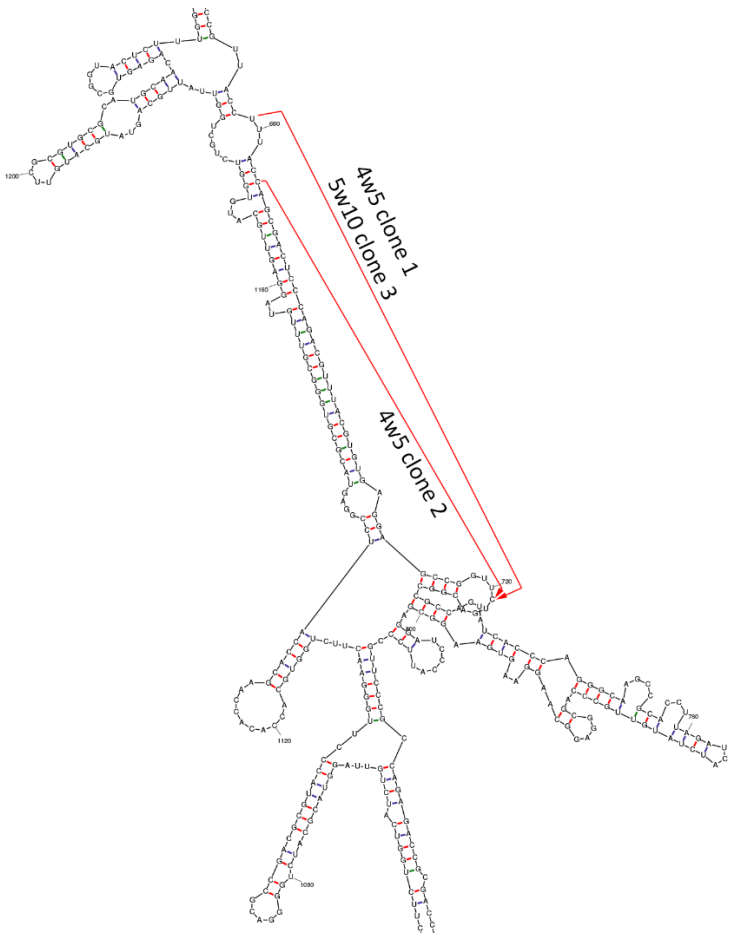
a) Overview of full-length 2.9kb PCR product including sequences unique for SAV3 FL-Δ6K and Helper-6K **b**) Deletions per single PCR product clone relative to full-length 2.9kb product are marked as lines, dots represents deletions of <10nt.

Color-coding has been used to visualize fish individuals and color shades differentiating each clone, also numbered with digits. Clone numbers in bold is used on clones with no deletions. Asterisks denotes deletion similar to earlier published in Petterson et al. 2013

Figure 4 | Examples of RNA secondary structures.



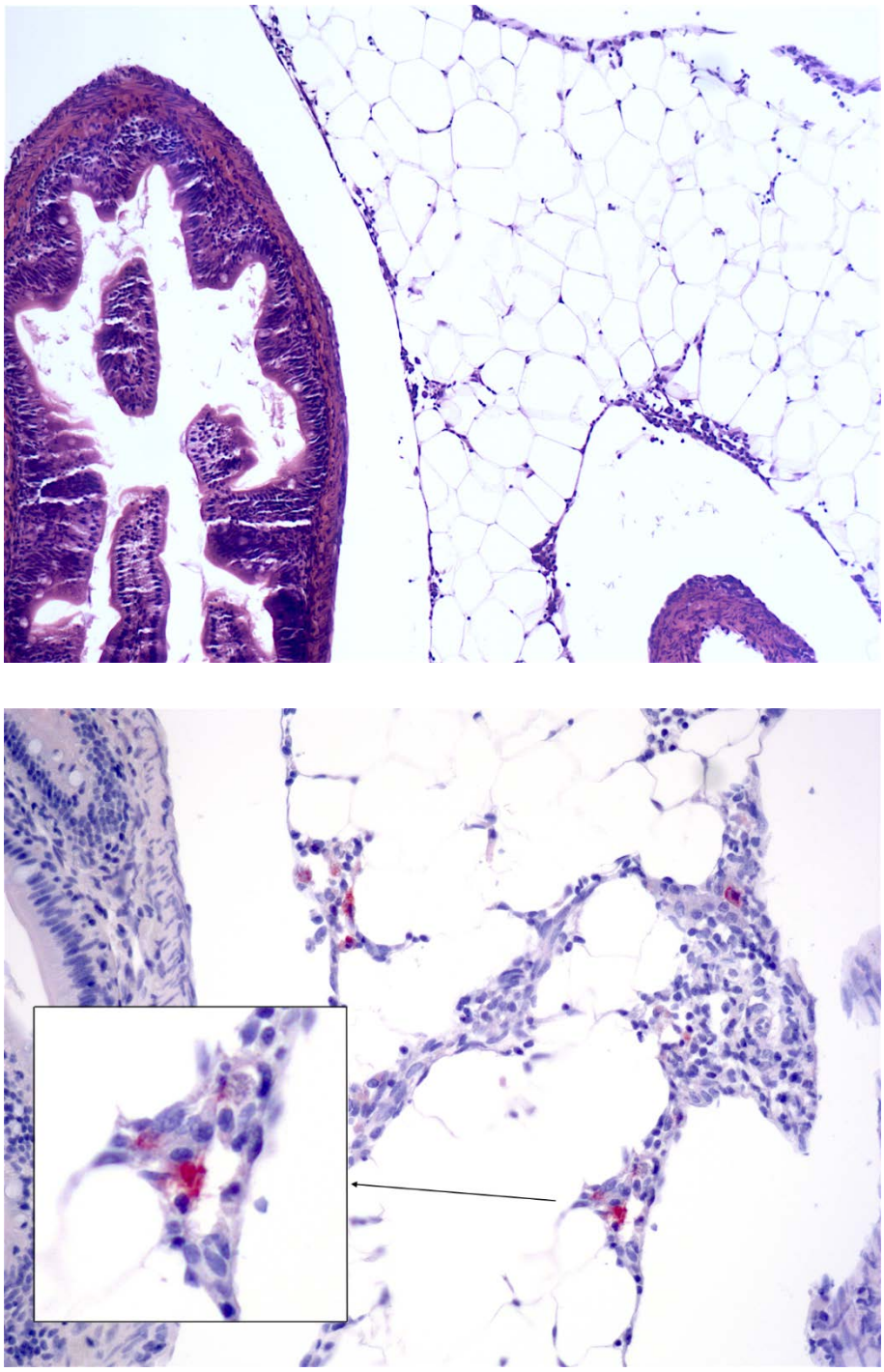
a) Secondary structure of 6K and surrounding RNA. Examples of positions for polymerase release and reattaching and relation to deletions in sequenced clones (Sequence alignment shown in Supplementary Fig. 3a) are indicated by arrows. A sequence covering 200bp in 3'end of E2 gene, 6K gene and 200bp in 5' end of E1 was submitted for predictions.



b) Section of secondary structure of E2 RNA. Examples of positions for polymerase release and reattaching and relation to deletions in sequenced clones (Sequence alignment shown in Supplementary Fig. 3b) are indicated by arrows. A sequence covering full E2 gene was submitted for predictions. Both RNA secondary structures were predicted by Mfold Web Server (version 2.3 energies) with folding temperature at 15°C.

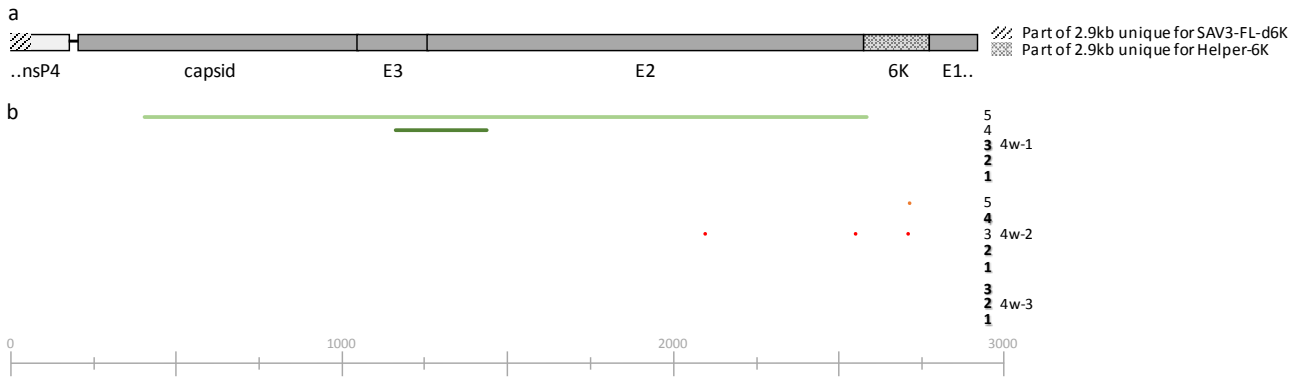
a)

b)



Supplementary Figure 1 | Pathological changes caused by recombined SAV3 in Atlantic salmon.

Histological tissue preparations of pancreas from Atlantic salmon injected with recombined SAV3, 3w-2H isolate, at 4 wpi. **a**) Loss of exocrine pancreas in perivisceral fat tissue. **b**) Immunohistochemistry of exocrine pancreatic tissue. Red coloration indicates presence of SAV3 antigen.



Supplementary Figure 2 |Sequencing of PCR products from recombined SAV3 injected fish.

a) Overview of full-length 2.9kb PCR product including sequences unique for SAV3 FL-Δ6K and Helper-6K

b) Deletions per single PCR product clone relative to full-length 2.9kb product are marked as lines, dots represents deletions of <10nt.

Color-coding has been used to visualize fish individuals and color shades differentiating each clone, also numbered with digits. Clone numbers in bold is used on clones with no deletions.

Supplementary Figure 3 |Sequence alignments.

a)

```
FL        TCGCCAACACGTTCAACCCGAACCCACCACCACTGACCGCACTGACTGCAGCATTGTGCTGCATACCTGGGGCTCGTGCGGACCAACCCTACCTGGACAT
5w7-3     TCGCCAACACGTTCAACCCGAACCCACCACCACTGACC----------------------------TGGGGCTCGTGCGGACCAACCCTACCTGGACAT
5w10-1    TCGCCAACACGTTCAACCCGAACCCACCACCACTGACCGCACTGACTGCAGCATTGCGCTGCATACCTGGGGCTCGTGCGGACCAACCCTACCTGGACAT
5w10-2    TCGCCAACACGTTCAACCCGAACCCACCACCACTGACCGCACTGACTGCAGCATTGTGCTGCATACCTGGGGCTCGTGCGGACCAACCCTACCTGGACAT
5w10-3    TCGCCAACACGTTCAACCCGAACCCACCACCACTGACCGCACT--------------------ACCTGGGGCTCGTGCGGACCAACCCTACCTGGACAT
4w5-1     TCGCCAACACGTTCAACCCGAACCCACCA------------------------------------------------------------------------
5w10-4    TCGCCAACACGTTCAACCCGAACCCA---------------------------------------------------------------------------
4w5-2     TCGCCAACA--------------------------------------------------------------------------------------------
```

```
FL        CATTGCCTACCTGTGGACCAACAGCAAAGTGGCCTTCGGGCTGCAATGCGCGGCGCCCGTGGCTTGCGTGCTCATCGTCACATACGCCCTTAGACACTGC
5w7-3     CATTGCCTACCTGTGGACCAACAGCAAAGTGGCCTTCGGGCTGCAATGCGCGGCGCCCGTGGCTTGCGTGCTCA------------------------
5w10-1    CATTGCCTACCTGTGGACCAACAGCAAAGTGGCCTTCGGGCTGCAATGCGCGGCGCCCGTGGCTTGCGTGCTCAT-----------------------
5w10-2    CATTGCCTACCTGTGGACCAACAGTAAAGTGGCCTTCGGGCTGCAATGCGCGGCGCCCGTGGCTTGCGTGCTCAT-----------------------
5w10-3    CATTGCCTACCTGTGGACCAACAGCAAAGTGGCCTTCGGGCTGCAATGCGCGGCGCCCGTGGCTTGCGTGCTCATC----------------------
4w5-1     ------------------------------------------------------------------------------------------------
5w10-4    ------------------------------------------------------------------------------------------------
4w5-2     ------------------------------------------------------------------------------------------------
```

```
FL        AGACTGTGCTGCAAGTCTTTTTTAGGGGTAAGAGGGTGGTCAGCTCTGCTGGTCATCCTTGCGTATGTACAGAGCTGCAAGAGCTACGAACACACCGTGG
5w7-3     ------------------------------------------------------------------CAGAGCTGCAAGAGCTACGAACACACCGTGG
5w10-1    ----------------------------------------------------------------AGAGCTGCAAGAGCTACGAACACACCGTGG
5w10-2    ----------------------------------------------------------------AGGGCTGCAAGAGCTACGAACACACCGTGG
5w10-3    ----------------------------------------------------------------AGAGCTGCAAGAGCTACGAACACACCGTGG
4w5-1     ------------------------------------------------------------------------------------------------
5w10-4    ------------------------------------------------------------------------------------------------
4w5-2     ------------------------------------------------------------------------------------------------
```

```
FL        TGGTCCCAATGGACCCGAGAGCCCCGTCGTACGAAGCAGTGATAAACCGGAATGGGTATGATCCCTTGAAGCTGA
5w7-3     TGGTCCCAATGGACCCGAGAGCCCCGTCGTAC-AAGCAGTGATAAACCGGAATGGGTATGATCCCTTGAAGCTGA
5w10-1    TGGTCCCAATGGACCCGAGAGCCCCGTCGTACGAAGCAGTGATAAACCGGAATGGGTATGATCCCTTGAAGCTGA
5w10-2    TGGTCCCAATGGACCCGAGAGCCCCGTCGTACGAAGCAGTGATAAACCGGAATGGGTATGATCCCTTGAAGCTGA
5w10-3    TGGTCCCAATGGACCCGAGAGCCCCGTCGTACGAAGCAGTGATAAACCGGAATGGGTATGATCCCTTGAAGCTGA
4w5-1     ----------------------------------------ACCGGAATGGGTATGATCCCTTGAAGCTGA
5w10-4    -------------------------------------AACCGGAATGGGTATGATCCCTTGAAGCTGA
4w5-2     ---------------------------------------------------------TTGAAGCTGA
```

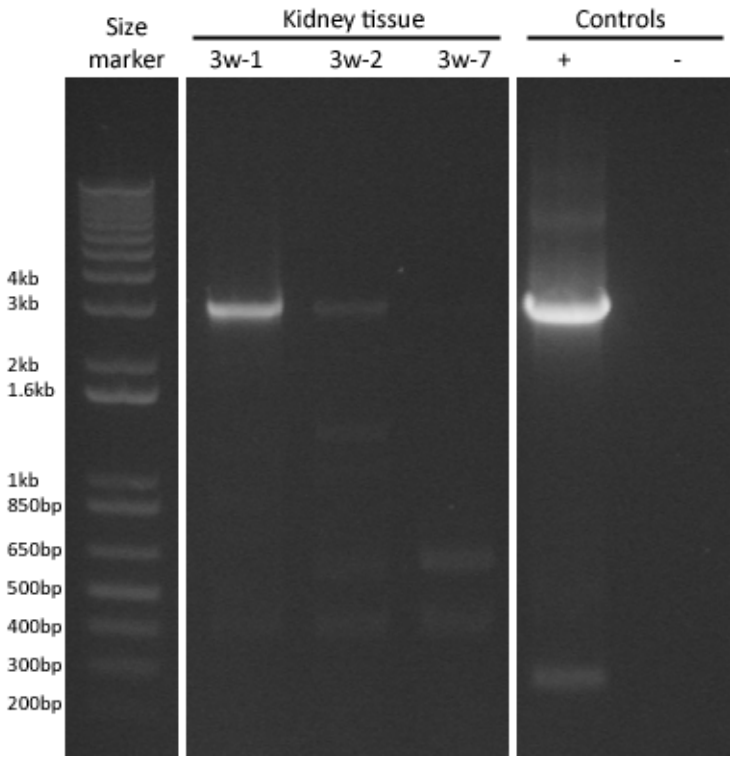
a) Alignments of example sequences from clones of 2.9kb amplicon showing deletions in 6K and surrounding RNA presented with predicted secondary structure in Fig 4a. FL – full length sequence.

b)

```
FL        CCGTTACCTTTACCAGCGACTCCCAGACGTTTACGTGTGAGGAGCCGGTTCTGACGGCCGCCAGTATCACCCAGGGCAAGCCGCACCTTAGATCATCTAT
4w5-1     CCGTTACCT-----------------------------------------CTGACGGCCGCCAGTATCACCCAGGGCAAGCCGCACCTTAGATCATCTAT
5w10-3    CCGTTACCT-----------------------------------------CTGACGGCCGCCAGTATCACCCAGGGCAAGCCGCACCTTAGATCATCTAT
4w5-2     CCGTTACCTTTACC------------------------------------CTGACGGCCGCCAGTATCACCCAGGGCAAGCCGCACCTTAGATCATCTAT


FL        GTTGCCCAGCGGAGGCAAGGAAGTGAAGGCGAGGATCCCATTCCCGTTCCCGCCAGAGACCGCGACC
4w5-1     GTTGCCCAGCGGAGGCAAGGAAGTGAAGGCGAGGATCCCATTCCCGTTCCCGCCAGAGACCGCGACC
5w10-3    GTTGCCCAGCGGAGGCAAGGAAGTGAAGGCGAGGATCCCATTCCCGTTCCCGCCAGAGACCGCGACC
4w5-2     GTTGCCCAGCGGAGGCAAGGAAGTGAAGGCGAGGATCCCATTCCCGTTCCCGCCAGAGACCGCGACC
```

b) Alignments of example sequences from clones of 2.9kb amplicon showing deletions in a region of E2 presented with predicted secondary structure in Fig 4b. FL – full length sequence.



Supplementary Figure 4 | Deletions illustrated as a ladder of PCR products visualized by gel electrophoresis

PCR products amplified from primers for 2.9kb amplicon were visualized on an agarose gel for individual 3w-1, 3w-2 and 3w-7 kidney tissue. Several shorter products than the expected 2.9kb size are shown for 3w-2 and 3w-7.

Supplementary table | Primers used for PCR reactions and genome sequencing

Primers used for PCR reactions	**Primer sequence (5´to 3´)**	**Annealing**
IPNV For	TGGCATTCTTGTTTGTTTCCT	58°C
IPNV Rev	CGTCCCGTTCAGAGCATAGA	
Ins For	AAGTGGAAAGCTGGTACAGAGTGGG	59°C
Ins Rev	GAGCTGTAGTTGGTGAGATGACG	
6K For	GAGCTGTAGTTGGTGAGATGACG	58°C
E1 For	TATCCGGATTCTTTTAGAGC	59°C
E1 Rev	GTAGAGATCTGTTCGGCAAT	
Primers used for sequencing of 2.9kb		
Ins For	See above	
S2	ACATCAAGTTCCAGGTCGCCGA	
S3	GTTCTCAATCGGGAGTGACCGCTAA	
S4	TGACCGCACTGACTGCAG	
Ins Rev	See above	
Primers used for full genome sequencing of 5th passage		
F1-For *	CCGCCGGCACTACAGTCACTGTA	60°C
F1-Rev*	ACTGCCCAACAGGTGTTACGCTTC	
F1-1	ACAAGCTGAAAAGCTGGCACCTG	
F1-2	AACGTCAACTCCATGGAT	
F1-3	GTGTTGCGCACCAGGAAA	
F1-4	AAAATAGCCGCACGTACG	
F2-For *	ATAATGAGCTCATGACTGCGGCTGC	60°C
F2-Rev *	CTCTTGTTCTTTTGAGTGGGGCGC	
F2-1	CCGCTGATCCCTATACTC	
F2-2	CAGTTGACAGATGGGACC	
F2-3	CCCTCAACTGACCGACAT	
	AAGAGGAAGAGGAACATG	
F2-5	GCACAGCCACGAGCCGCC	
F3-For *	GCCTGCAGAGACGAAGAAGAAGCT	60°C
F3-Rev*	GTCCCTTCCCAGGTATCGCAGAG	
F3-1	ATGGCCGCCACCAAGCGC	
F3-2	GAAGACCTCCTGACCCTC	
F3-3	CGCGCGAGGGGAGTACGT	
F3-4	GCCGAGGACATGGACCTC	
F4-For *	TACGACACACAAATCCTCGCCG	60°C
F4-Rev*	GCGGCACTTCTTCACCACGCAGT	
F4-1	CCGTGCTGCTGACTACACGGA	
F4-2	GTGGCTGCCACCGTCTCT	

* Primers also used for PCR amplification of fragment 1-4.