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RESEARCH ARTICLE

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Genetically modified attenuated salmonid alphavirus: A potential strategy for immunization of Atlantic salmon

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Abstract

Pancreas disease (PD) is a serious challenge in European salmonid aquaculture caused by salmonid alphavirus (SAV). In this study, we report the effect of immunization of Atlantic salmon with three attenuated infectious SAV3 strains with targeted mutations in a glycosylation site of the envelope E2 protein and/or in a nuclear localization signal in the capsid protein. In a pilot experiment, it was shown that the mutated viral strains replicated in fish, transmitted to naïve cohabitants and that the transmission had not altered the sequences. In the main experiment, the fish were immunized with the strains and challenged with SAV3 eight weeks after immunization. Immunization resulted in infection both in injected fish and 2 weeks later in the cohabitant fish, followed by a persistent but declining load of the mutated virus variants in the hearts. The immunized fish developed clinical signs and pathology consistent with PD prior to challenge. However, fish injected with the virus mutated in both E2 and capsid showed little clinical signs and had higher average weight gain than the groups immunized with the single mutated variants. The SAV strain used for challenge was not detected in the immunized fish indicating that these fish were protected against superinfection with SAV during the 12 weeks of the experiment.

KEYWORDS

attenuation, capsid, N-glycosylation, pancreas disease, salmonid alphavirus, virulence

1 | INTRODUCTION

Salmon pancreas disease virus (SPDV) is the etiological agent of pancreas disease (PD) and sleeping disease in farmed Atlantic salmon *(Salmo salar)* and rainbow trout *(Oncorhynchus mykiss)*, respectively. SPDV belongs to the genus *Alphavirus* in the family *Togaviridae*. The virus is hereafter referred to as salmonid alphavirus (SAV). Classically, alphaviruses can be divided into two phylogenetic clades, the Old world and New world viruses, which roughly reflect their geographical dispersal (Levinson et al., 1990). They can be found in a large range of vertebrate animals and are transmitted by arthropod vectors. SAV was separated from the other alphaviruses before the Old world–New world split (Karlsen et al., 2010; Villoing et al., 2000), and it is the most divergent alphavirus known to date regarding phenotypical features, genetic distance and ecological niche (McLoughlin & Graham, 2007). Where other alphaviruses depend on an invertebrate vector, SAV is transmitted in the absence of biological vectors (Kongtorp et al., 2010). The early divergence of

SAV makes it particularly informative in studies of alphavirus' evolution, and properties common for SAV and other alphaviruses are likely to have existed in ancestral alphaviruses.

The main hosts of SAV are salmonid fish, but SAV has also been isolated from non-salmonid marine fish species (McCleary et al., 2014). In Norway, PD is caused by SAV subtypes 2 and 3 and only found in the seawater phase of farmed Atlantic salmon. Fish with acute PD are typically lethargic and show initially necrosis followed by inflammation of pancreatic tissue, heart and skeletal muscles (McLoughlin et al., 2002; Xu et al., 2012).

Systematic and comprehensive testing, rigorous management and vaccination of fish in PD enzootic areas have not been sufficient to curb the number of outbreaks (Jensen et al., 2012; Skjold et al., 2016). Inactivated whole-virus PD vaccines are commonly used, but recently, a DNA vaccine was introduced to the market (Dalmo, 2018).

Many alphavirus proteins are important for the viral virulence (Akhrymuk et al., 2012; Cruz et al., 2010; Fros et al., 2013; Galbraith et al., 2006; Johnson et al., 1986; Jupille et al., 2011; Simmons et al., 2010). Mutations in the surface E2 glycoprotein of SAV that supposedly change receptor binding properties have been associated with altered virulence (Mérour et al., 2013). Studies have shown that alterations of glycosylation motifs, and thus prevention of glycan attachment, may alter the infectivity of alphaviruses (Knight et al., 2009; Nelson et al., 2016). Previously, we have shown that a mutation in the predicted N-linked glycosylation site in E2 attenuated SAV3 replication properties in cell culture, causing less cytopathogenic effects and production of infectious virus, while similar mutations in glycosylation site in E1 inactivated the virus (Aksnes et al., 2020). In addition, the capsid has been shown to be associated with inhibition of cellular proliferation, assumed to be important for the cytopathic effect caused by SAV (Karlsen, Yousaf, et al., 2010). This suggests that the SAV capsid protein is an important factor for pathogenesis during infection. Interestingly, subcellular localization studies suggest that the capsid proteins of many alphaviruses, including SAV, partly localize to the nucleus even though alphavirus genome replication occurs entirely within the cytoplasm (Aguilar et al., 2007, 2008; Favre et al., 1994; Garmashova, Gorchakov, et al., 2007; Jalanko & Soderlund, 1985; Karlsen, Yousaf, et al., 2010; Michel et al., 1990; Mitchell et al., 1997). Nucleo-cytoplasmic trafficking is critical for many cellular processes. Cargos to be transported across the nuclear envelope contain a variety of different sequence motifs that are called nuclear localization signals (NLS) and nuclear export signals (NES) (Strom & Weis, 2001). Alphaviruses have the ability to interfere with cellular nuclear–cytoplasmic transport, and this property is associated with the N-terminal part of the capsid protein that contains an NLS and a leucine-rich NES. Similar putative NLS and NES motifs are present in the SAV capsid (Karlsen, Yousaf, et al., 2010). By introducing several amino acid substitutions downstream of the NES and within the NLS, the ability of the capsid to cause transcriptional shutoff of Venezuelan equine encephalitis virus (VEEV) could be inhibited (Atasheva et al., 2010).

Live, attenuated vaccines are considered the gold standard for preventing viral diseases in mammals (Minor, 2015). A challenge is to keep the balance between reduced virus replication and associated pathologic lesions, while retaining antigenicity and induction of a protective immune response. However, regarding the potential use of live-attenuated vaccine technology in marine fish farming, not efficiency, but safety issues is considered being the main challenge. The open structure of modern marine salmon farming and interaction with surrounding fauna makes safety issues particularly important. In general, live-attenuated vaccines may regain virulence through back-mutation or compensatory mutations elsewhere in the genome. Consideration must be taken to reduce the probability of reversion to virulence by for example generation of deletions in the genome, introduction of numerous mutations, as well as estimating the stability of the introduced changes.

In the present study, in the search of possible robust live-attenuated virus vaccines, variants of SAV with targeted genetical modifications causing cell culture attenuation were experimentally injected in fish. Several SAV3 infectious strains with targeted genetical modifications were constructed. Three different strategies were used, one targeting the subcellular localization signals of the capsid protein, the second targeting a predicted N-linked glycosylation motif in the E2 protein and the third was a combination of these. The differently mutated infectious SAV3 strains were tested in an experimental immunization challenge in Atlantic salmon.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Two in vivo experiments were conducted at the Aquaculture Research Station, Tromsø, Norway. The challenge trials were approved by the Norwegian Animal Research Authority (NFDA) according to the European Union Directive 2010/63 /EU (permit numbers 16409 and 19014) and were performed in accordance with the recommendations of the current animal welfare regulations: FOR-1996-01-15-23 (Norway).

2.2 | Computer analyses

Multiple sequence alignment of capsid protein sequences from the SAV3, Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV), Chikungunya virus (CHIKV), Ross River virus (RRV), Semliki Forest virus (SFV) and Sindbis virus (SINV) was performed using AlignX software (Vector NTI Advance™ 11, Invitrogen, Carlsbad, CA, USA). Nuclear export signals (NESs) and nuclear localization signals (NLSs) in the SAV3 capsid protein were predicted using NetNES 1.1 (La Cour et al., 2004) and PSORT II (https://www.genscript.com/psort/psort2.html), respectively. Protein secondary structure predictions were performed using PSIPRED 4.0 (Jones, 1999). We have previously predicted the N-glycosylation

site in SAV3 E2 using the NetNGlyc 1.0 server (available at http://www.cbs.dtu.dk/services/NetNGlyc/) supplemented by data from structure homology modelling of the protein using iTasser (Aksnes et al., 2020).

2.3 | Cells

Chinook salmon embryo cells (CHSE-214) (RRID: CVCL_278) and Chum salmon heart cells (CHH-1) (RRID: CVCL_4143) were used. Both cell lines were cultivated at 20°C in grow out medium: Leibovitz (L-15) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2-mercaptoethanol (40 μM) and gentamicin–sulphate (50 μg/ml) (all from Life Technologies, Paisley, Scotland, UK) or in maintenance medium containing 2% FBS.

2.4 | Plasmid constructs

A plasmid containing the entire SAV3 genome (prSAV3) was used as template for the construction of mutated infectious strains. The prSAV3 was originally cloned from the wild-type SAV3 isolate H20/03 (DQ149204, AY604236) (Karlsen et al., 2010). Single-site substitutions were done in prSAV3 using QuickChange site-directed mutagenesis (Agilent, Santa Clara, CA, USA) following the manufacturer's instructions. The primers used in the mutagenesis (Table 1) were designed with the QuickChange Tm calculator (available at https://www.agilent.com/store/primerDesignProgram.jsp). Five different SAV3 infectious cDNA strains that were altered in the capsid and/or the E2 coding sequences were generated (Table 2). In the capsid, slightly downstream of a predicted nuclear export signal (NES), the four amino acids Gln-Ala-Arg-Val (QARV) at positions 66–69 relative to the start of the capsid protein sequence were substituted with four glycines (GGGG). Additional mutations were introduced in the capsid by substituting two lysines (K) with alanines (A) in a predicted bipartite nuclear localization signal (NLS) at positions 79 and 81. In the E2 protein, an asparagine (N) was substituted with alanine (A) at position 319 in a predicted N-linked glycosylation site as previously described (Aksnes et al., 2020). Also, a XbaI site originally introduced in the junction region of prSAV3 as a tag to distinguish between the infectious clone from that of wild-type SAV3 (Karlsen, Villoing, et al., 2010) was replaced with the wild-type sequence prior to the main challenge experiment in the present study.

CHSE-214 cells were transfected with 0.5 μg endotoxin-free plasmid and 100 μl Ingenio solution (Mirus Bio LLC, Madison, WI USA) was added to 4×10^6 CHSE-214 cells. The cells were transfected using the Amaxa Nucleofector device (Lonza, Basel, Switzerland), following the manufacturer's instructions. Transfected cells were transferred to 25-cm^2 flasks (Corning®), and the cells were incubated at 20°C for 24 hr before being transferred to 15°C and incubated for 14 days (passage 0, P0).

2.5 | Recovery and passage of mutated strains

Fourteen days after transfection, 1 ml of supernatant was transferred from flasks containing transfected CHSE-214 cells to CHH-1 cells (Passage one, P1). The CHH-1 cells were incubated at 15°C for one hour, added 5 ml maintenance medium and then incubated for 14 days. Following the incubation period, three serial passages of supernatant were performed (P2-P4). To quantify viral RNA from the P1-P4 supernatants, RT-qPCRs were performed as previously described (Aksnes et al., 2020). Viral RNA from infected cells (P4) was also Sanger-sequenced following PCR to verify that the mutations were present. Propagation and quantification ($TCID_{50}$) of isolates were performed with CHH-1 cells using standard techniques as previously described (Aksnes et al., 2020).

2.6 | Next-generation sequencing (NGS)

The complete genome sequence of the SAV3 strain H20/03, originally isolated from a PD outbreak in Norway in 2003 (Karlsen et al., 2006) (AY604236) and since passed an unknown number of times (>20) in cell cultures, was obtained by next-generation sequencing. Total RNA was isolated from CHH-1 cell supernatant infected with the H20/03 isolate using Trizol LS (Life Technologies, Carlsbad, California, USA) and RNeasy mini spin columns (Qiagen, Hilden, Germany), following the protocols recommended by manufacturers. Macrogen (Seoul, South Korea) performed DNase I

TABLE 1 Primers used to generate the mutations in the plasmid constructs

Primer	Sequence (5' → 3')[a]
$Cap_{66QARV→GGGG}$–For	GCTGGACTTGCCGGCG**GG**GG**A**G**G**GGGG**G**GATCGTCGTGGAC
$Cap_{66QARV→GGGG}$–Rev	GGTCCACGACGATC**C**CCC**C**C**T**CC**CC**CGCCGGCAAGTCCAGC
$Cap_{79K81K→AA}$–For	GTGGACCAAGACGTGTTCAG**GC**AAGC**GC**GCAGAAGAAGAAG
$Cap_{79K81K→AA}$–Rev	CTTCTTCTTCTGC**GC**GCTT**GC**CTGAACACGTCTTGGTCCAC
$E2_{319N→A}$–For	GAACCTTGGTTTCCACAGC**GCG**GCCACATCCGAATGGATCC
$E2_{319N→A}$–Rev	GGATCCATTCGGATGTGGC**CGC**GCTGTGGAAACCAAGGTTC
$rSAV3_{XbaI→wt}$–For	TCTGCATCATACTCT**CA**ACCAACCATGTTTCCC
$rSAV3_{XbaI→wt}$–Rev	GGGAAACATGGTTGGTTGAGA**GT**ATGATGCAGA

[a]Mutated nucleotides are shown in bold and underlined.

treatment and library preparation with the TruSeq Stranded Total RNA Library prep. kit (Illumina Inc., San Diego, CA, USA), and de novo sequencing using a HiSeq2500 system (2x100 PE). The paired-end reads were assembled using CLC Genomics Workbench (Qiagen), and the complete H20/03 genome was mapped using the SAV3 genome submitted under GenBank acc. no. JQ799139 as reference. Total read count obtained was 128,189,020 and 84% of the reads (107,716,522) mapped to the reference genome. The viral genome, renamed H20/03/2, has been submitted to GenBank (acc no. MW196361).

2.7 | In vivo *experiments*

2.7.1 | Pilot

Prior to the main in vivo experiment, a pilot study was conducted with infectious SAV3 strains constructed in a previous work (Aksnes et al., 2020) (Figure 1). The pilot was run at the Aquaculture Research Station, Tromsø, Norway, and the fish were held in sea water (34‰ salinity). Shedder fish were injected intraperitoneally (i.p.) at Day 0 with 0.2 ml cell culture medium containing 10^4 $TCID_{50}$ with a panel of recombinant SAV3 infectious strains: rSAV3, rSAV3-$E2_{319Q}$, rSAV3-$E2_{319A}$, rSAV3-Cap_{NLS} and rSAV3-Cap_{NES}. The XbaI site in the junction region was present in these strains. Cohabitants were introduced at Day 1. Blood, gill, spleen, kidney, heart, liver and pancreas were collected from sampled fish. To assess the stability of mutations, Sanger sequencing was performed from virus-positive cohabitants from the sampling at 6 weeks post-immunization (wpi). The pilot, originally intended to have a duration of 10 weeks, was terminated at week 7 due to technical issues. Hence, results after 6 wpi are not shown.

TABLE 2 Plasmid constructs transfected into CHSE-214 cells

Plasmid	Mutation	Tentative interaction
prSAV3	–	Original clone
prSAV3-Cap_{NLS}	$Cap_{79A, 81A}$	Intracelluar placement
prSAV3-$Cap_{NES, NLS}$	$Cap_{66–69GGGG', 79A, 81A}$	Intracelluar placement
prSAV3-$E2_{319A}$	$E2_{319A}$	Receptor binding protein
prSAV3-$E2_{319Q}$	$E2_{319Q}$	Receptor binding protein
prSAV3-Cap_{NLS} $E2_{319A}$	$Cap_{79A, 81A}$ $E2_{319A}$	Intracelluar placement
prSAV3-$Cap_{NES, NLS}$ E_{319A}	$Cap_{66–69GGGG'79A,81A}$ $E2_{319A}$	Intracelluar placement

2.8 | Main in vivo experiment

Four infectious SAV3 strains (Figure 2) were used in the main in vivo trial. The fish were non-vaccinated Atlantic salmon, presmolts, strain NLA reared in hatchery at the research station and confirmed free of the salmon pathogens ISAV, SAV, PRV and IPNV by RT-qPCR. They were kept in running freshwater at 10°C, exposed to continuous light and fed with commercial dry feed and starved for 24 hr prior to handling and sampling. The fish were randomly selected for immunization, anaesthetized by bath immersion in benzocaine chloride (0.5 g/10 L) (2–5 min), labelled (tattoo) and intramuscularly (i.m.) injected with the viruses. The fish were observed minimum once per day, and subjective observation of clinical signs was tentatively assessed. Prior to handling and sampling, the fish were anaesthetized by bath immersion in benzocaine chloride.

2.9 | Design

Fish were observed daily throughout the 12-week duration of the experiment (Figure 1). In tanks 1–4, thirty-nine fish were injected

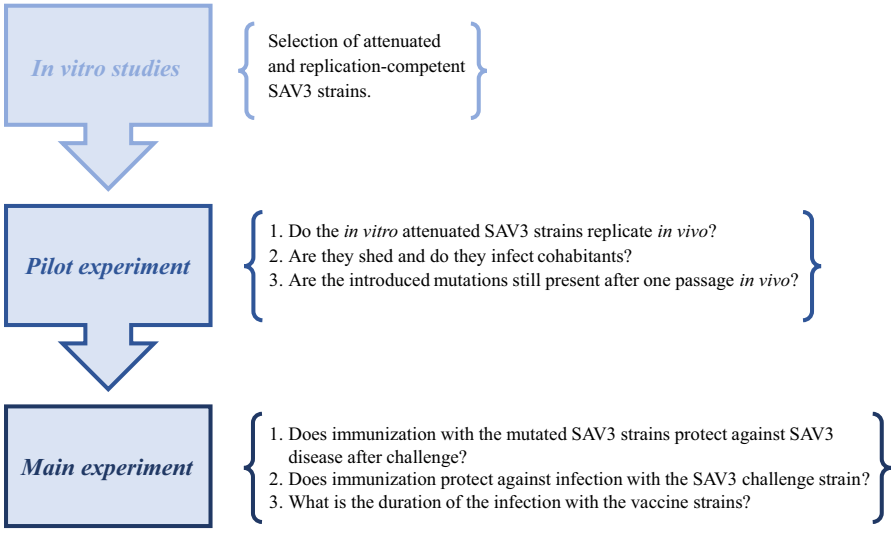


FIGURE 1 Order of the experiments performed: in vitro testing of mutated SAV3 strains, pilot in vivo experiment and main in vivo experiment (see main text, Table 2 and Figure 2 for details regarding the mutated strains) [Colour figure can be viewed at wileyonlinelibrary.com]

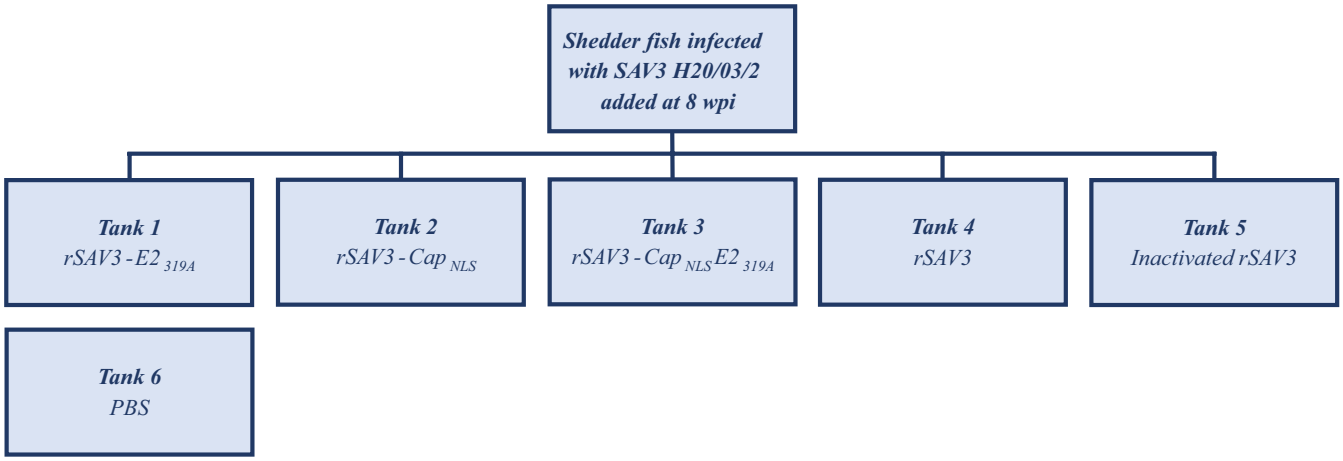


FIGURE 2 Experimental set-up of the main in vivo experiment. The experiment lasted for 12 weeks and sampling was done at 2, 4, 6, 8, 10 and 12 wpi. SAV3 shedders were added at 8 wpi. No shedder fish were added to Tank 6 (negative control) [Colour figure can be viewed at wileyonlinelibrary.com]

i.m. at Day 0 with 0.2 ml cell culture medium containing 10^2 TCID50 virus, that is rSAV3-$E2_{319A}$, rSAV3-Cap_{NLS}, rSAV3-$Cap_{NLS}E2_{319A}$ and rSAV3, respectively. Twenty cohabitant fish were introduced at Day 1. Tank 5 contained fish-injected i.m. with a vaccine based on inactivated SAV3 with water-in-oil adjuvant (Karlsen et al., 2012) (kindly supplied by PHARMAQ AS). Tank 6 fish contained control fish that were injected with PBS. Shedder fish kept in a separate tank were injected with H20/03/2 at week 7, and after sampling at week 8, twelve shedder fish were placed in tanks 1–5. Six fish per injected group and three fish per cohabitant group were sampled at 2, 4, 6, 8, 10 and 12 wpi.

Blood samples were collected by caudal venipuncture and kept on ice overnight to allow separation of plasma from the clot. Samples were then clarified by centrifugation and the plasma was removed by aspiration and stored at − 80°C for further use. Spleen, kidney, liver, heart, pancreas and gill (second arch) were sampled for histology and fixed in 10% phosphate-buffered formalin. After 24 hr, the formalin was replaced with 70% ethanol and samples stored at 4°C until further use. For RT-qPCR analyses, spleen, kidney, liver, heart and pancreatic tissue from each fish were placed in 1 ml of RNAlater (Qiagen).

Weight and length of sampled fish were recorded throughout the experiment. An overview of the main trial is shown in Figure 1.

2.10 | RNA isolation and RT-qPCR

Total RNA was extracted from heart tissue using the RNeasy Mini kit and QIAcube System (110 V). Prior to extraction, 10–30 mg tissue was transferred to safe-lock tubes containing 500 µl RLT buffer and a 5-mm stainless steel bead (Qiagen). Homogenization was carried out by high-speed shaking, 25 Hz for 2 x 5 min, in a TissueLyser (Qiagen). The lysate was cleared by centrifugation at 15,000 rpm for 3 min. The concentration of RNA was determined by spectrophotometry using the Nanodrop ND1000 (Nanodrop Technologies, Wilmington, USA). For each sample, 750 ng of total RNA was subjected to cDNA synthesis using Quantitech® Reverse Transcription (Qiagen), in a total volume of 20 ul. The TaqMan assay (PE Applied Biosystems) was used for qPCR with an input of 5 µl diluted cDNA (equivalent to15 ng RNA used in cDNA synthesis) per reaction in a total reaction volume of 13 µl. Primers and probe targeting SAV gene segment nsP1 were used (Hodneland & Endresen, 2006). The qPCR conditions were 300 nM primer, 200 nM probe, 6.5 µl TaqMan® Gene Expression Master Mix and 2.3 µl RNase-free water. The cycling parameters were 50°C/2 min and 95°C/10 min, followed by 40 cycles of 95°C/15 s, 58°C /15s and 60°C/1 min in an AriaMx (Agilent, Santa Clara, CA, USA).

The relative expression of Mx and viperin was examined at 2 and 4 wpi. The primers and probe targeting Mx mRNA were ssMx_F: 5'-GATGCTGCACCTCAAGTCCTATTA-3', ssMx_R: 5'-CACCAGGTAGCGGATCACCAT-3' and 5'(6-FAM)-CTGATCAGCCAAACGTTGACTGGATATCCT-TAMRA'3 (modified from (McBeath et al., 2007) and for viperin ssVip_F: 5'CCGGAAGTACAAAGTGGCATTCAAA'3, ssVip_R 5'CTGGTCACTGATGTTTTCTCTCATGT'3 and ssVip_probe: 5' (6-FAM)-TTAACTCTGTGATCAACACCT-MGBNFQ. Primer and probe concentrations for Mx were 300 nM and 200 nM, respectively, and for viperin 900 nM and 200 nM, respectively. The cycling parameters were 50°C/2 min and 95°C/10 min, followed by 40 cycles of 95°C/15 s, 58°C /15s and 60°C/1 min.

2.11 | Histopathology

The formalin–ethanol-fixed heart tissues were processed in a Thermo Scientific Excelsior® tissue processor and embedded in paraffin Histowax using a Tissue-Tek®, TEC 5 (Sakura) embedding centre. Embedded tissue was sectioned at 1,5–2 µm using a Leica RM 2,255 Microtome, sections were mounted on glass slides and stained with haematoxylin–eosin (HE) (Histolab products AB). The stained

Score	Description
0	No lesions, normal
1	Focal acute myocardial degeneration/necrosis < 7 fibres affected
2	Multifocal myocardial degeneration/necrosis ± inflammation (<15% of ventricle affected)
3	Multifocal myocardial degeneration/necrosis ± inflammation (>15 & <50% of ventricle affected)
4	Severe diffuse myocardial degeneration/necrosis ± inflammation (>50% of ventricle affected)

TABLE 3 Semi-quantitative scoring system for evaluating PD/SAV-associated heart lesions on a scale from 0 to 4, where score 0: no lesions; score 1: very mild lesions; score 2: mild lesions; score 3: moderate lesions; score 4: severe lesions

FIGURE 3 Location of nuclear export signals (NES) (red boxes) and nuclear localization signals (NLS) (green boxes) in the capsid protein sequence of SAV3 and Venezuelan equine encephalitis virus (VEEV). Black triangles indicate four substitutions to glycine downstream of the NES and black boxes substitutions to alanine in the NLS. Data from VEEV were used as a model for the substitutions (Atasheva et al., 2010, 2015). Alphavirus sequences used were VEEV (P09592.2), Eastern equine encephalitis virus (EEEV, ANB41743.1), Chikungunya virus (CHIKV, AEA10291.1), Ross River virus (RRV, P08491.3), Semliki Forest virus (SFV, NP_463458.1) and Sindbis virus (SINV, CAA24684.1). The position numbering is according to the SAV3 sequence. Additional putative SAV3 bipartite NLS (R_{255}–R_{271}) and NES (L_{169}–L_{182}) are not shown (Karlsen, Yousaf, et al., 2010) [Colour figure can be viewed at wileyonlinelibrary.com]

slides were scanned in an Aperio Scan Scope AT Turbo slide scanner and read using Aperio ImageScope v12.3.2.8013 (Leica). Hearts from the six different treatment groups (36 hearts in total) sampled 6 wpi were subjected to histopathologic analysis. The lesions in each heart sample were scored in a blinded fashion by an experienced pathologist, in a semi-quantitatively manner, using a modified scoring system according to McLoughlin et al. (2006) (Table 3).

2.12 | Statistical analysis

One-way ANOVA was performed using JMP Pro 15 for windows (JMP Software, Marlow, United Kingdom). The significant level for rejection of null hypothesis (H_0) was set at probability value (p) < 0.05.

3 | RESULTS

3.1 | Prediction of SAV3 nuclear export and import signals

Predictions of NES and NLS in the SAV3 capsid protein and N-glycosylation sites in E2 have been done previously (Aksnes et al., 2020; Karlsen, Yousaf, et al., 2010). Our analyses confirmed two bipartite NLSs located at R_{71}-Q_{100} and R_{255}-R_{271} (Figure 3) and the NES predicted in NetNES located between L_{169} and L_{182} (not shown) when the complete capsid protein sequence was used as input (Karlsen, Yousaf, et al., 2010). For NES, when analysing the N-terminal part of the capsid protein, NetNES predicted an additional motif between M_{53}-L_{58} and possibly extending to I_{46}-L_{63}, partially corresponding with the NES in the capsid protein in VEEV (Atasheva et al., 2010) (Figure 3). The E2 N-glycosylation site is predicted to be at N_{319} (Aksnes et al., 2020).

The alphavirus capsid protein sequences are less conserved in the N-terminal half compared with their C-terminal half, and this also includes the SAV capsid protein, although overall less conserved compared with the other alphaviruses (data not shown). Secondary

TABLE 4 Plasmid constructs and recovered viruses

Plasmid	Recovery	Virus
prSAV3	Yes	rSAV3
prSAV3-Cap_{NLS}	Yes	rSAV3-Cap_{NLS}
prSAV3-$Cap_{NES,NLS}$	No	–
prSAV3-$E2_{319A}$	Yes	rSAV3-$E2_{319A}$
prSAV3-$E2_{319Q}$	Yes	rSAV3-$E2_{319Q}$
prSAV3-$Cap_{NLS}E2_{319A}$	Yes	rSAV3-$Cap_{NLS}E2_{319A}$
prSAV3-$Cap_{NES,NLS}E2_{319A}$	No	–

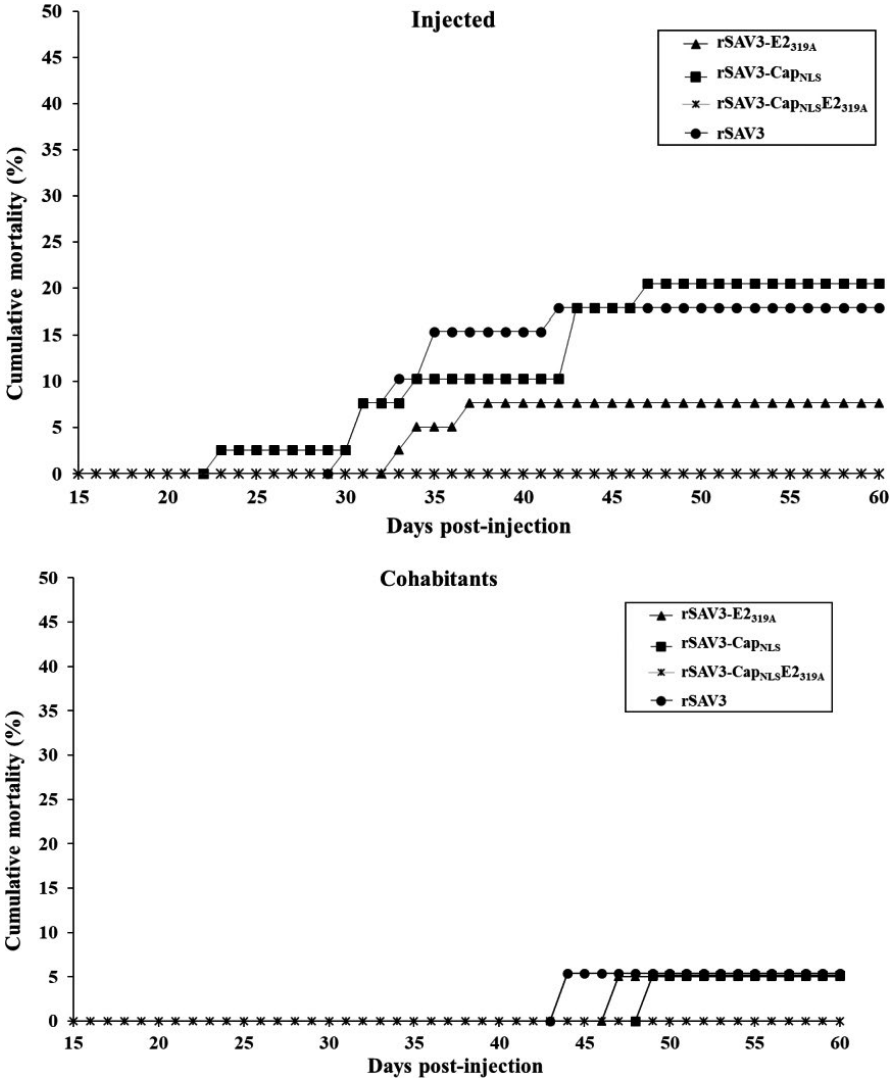


FIGURE 4 Cumulative mortalities for injected and cohabitant fish exposed to the infectious strains rSAV3, rSAV3-$E2_{319A}$, rSAV3-Cap_{NLS} and rSAV3-$Cap_{NLS}E2_{319A}$. No mortalities occurred in the groups injected with inactivated SAV3 vaccine and PBS (not shown)

structure predictions show a high level of structural conservation of the SAV3 capsid protein, including the N-terminal helix I, compared with other alphaviruses (Figure S1).

3.2 | Recovery of infectious strains

In addition to the non-mutated rSAV3, four mutated SAV3 viruses were recovered: rSAV3-$E2_{319A}$, rSAV3-$E2_{319Q}$, rSAV3-Cap_{NLS} and rSAV3-$Cap_{NLS}E2_{319A}$ (Table 4). No viable virus particles were recovered for the clone with double mutation in the capsid, rSAV3-$Cap_{NES, NLS}$, or the clone carrying all three mutations, rSAV3-$Cap_{NES, NLS}E2_{319A}$ (Table 4).

3.3 | Comparison of prSAV3 and H20/03/2 genome sequences

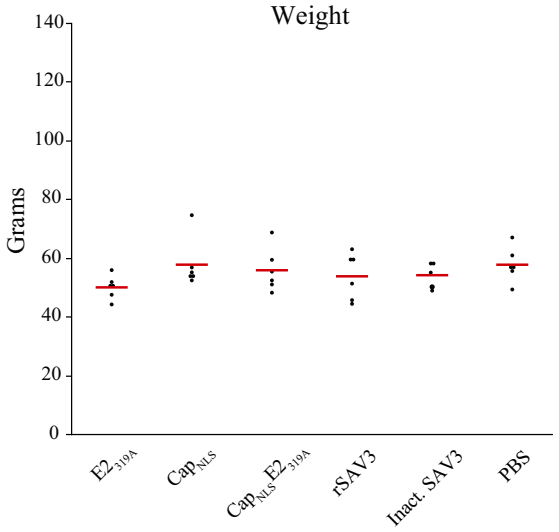
Comparison of the full-length sequences of the prSAV3 cDNA clone and H20/03/2 revealed 12 nucleotide differences (Table S1) where four of these caused amino acid changes. In prSAV3, these four encoded amino acids have been reported in other field isolates (AY604235-38, KC122918-26 and JQ799139). In H20/03/2, on the other hand, these residues have not been found in available SAV3 sequences in GenBank.

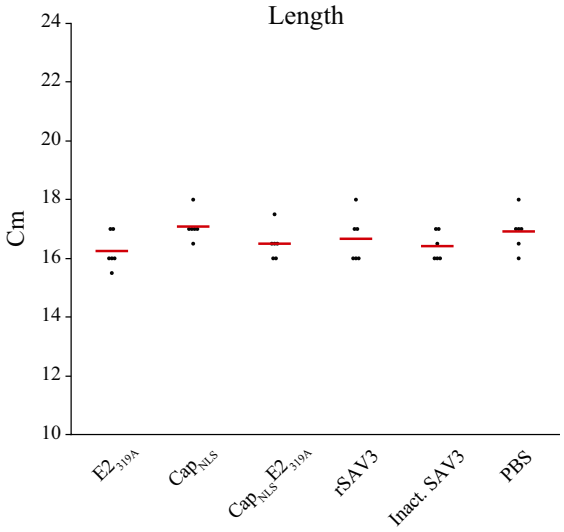
3.4 | Pilot study

The purpose of the pilot study was to investigate the ability of the infectious SAV3 strains to replicate in injected fish, transmit to cohabitant fish and determine the stability of the introduced mutations following replication in vivo.

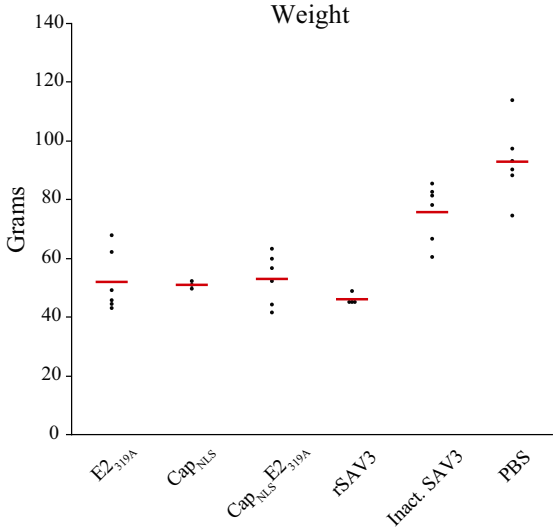
The fish in the pilot injected with the infectious strains were SAV3-positive as detected by RT-qPCR in heart for all in the injected fish at 1 wpi and in the cohabitants at 6 wpi. This confirmed that the infectious rSAV3 strains did replicate in fish and produced infectious particles that were shed and infected the cohabitant fish. The introduced mutations were verified by sequencing of viral RNA from cohabitant fish at 6 wpi. No reversion was observed following five

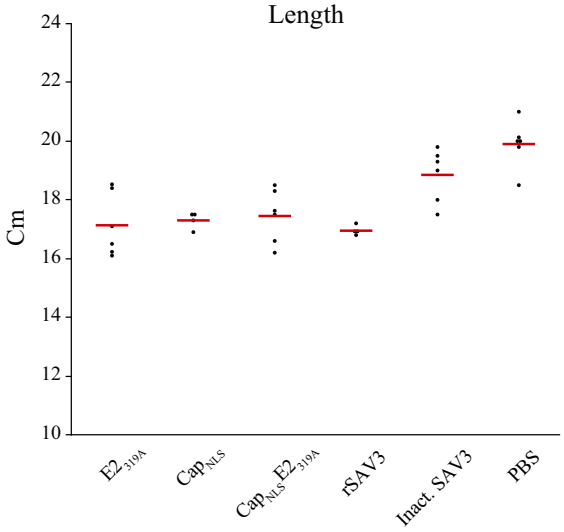
4 wpi





8 wpi





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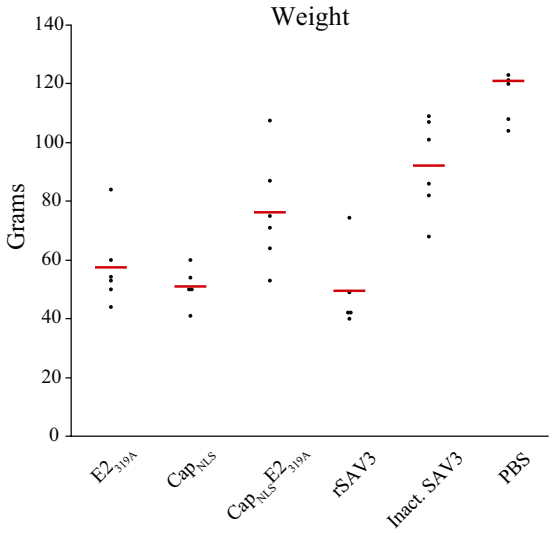

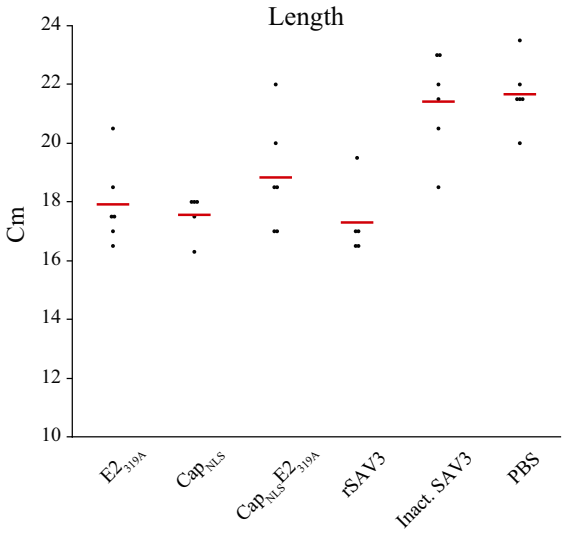

FIGURE 5 Weight and length of injected fish at 4 wpi, 8 wpi and 12 wpi. Red lines represent average values from sampled fish within each group. At 4 and 8 wpi, no significant differences were observed in weight between the groups immunized with mutated virus strains. At 12 wpi, the weight of rSAV3-$Cap_{NLS}E2_{319A}$-injected group was significantly higher than that for the other groups injected with mutated virus, while no significant differences were observed between rSAV3$Cap_{NLS}E2_{319A}$ and the inactivated SAV3-injected groups [Colour figure can be viewed at wileyonlinelibrary.com]

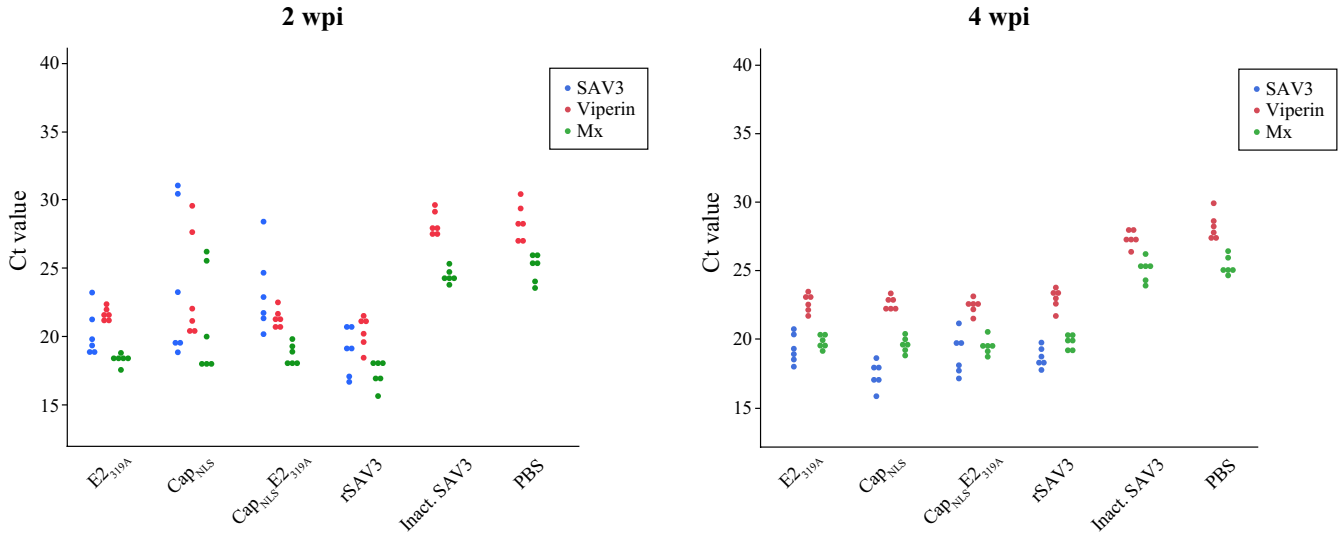

FIGURE 6 Comparison of expression levels between nsP1, Mx and viperin in heart at 2 wpi and 4 wpi. At 2 wpi, differences in Mx and viperin expression levels were high within the groups. At 4 wpi, Mx and viperin expression levels from inactivated SAV3- and PBS-injected fish differ significantly from fish injected with infectious virus [Colour figure can be viewed at wileyonlinelibrary.com]

in vitro passages and two passages in vivo suggesting stability of the introduced mutations.

We observed no significant differences in mortality and morbidity when comparing the results for the viruses having either the $E2_{319A}$ or $E2_{319Q}$. Only the $E2_{319A}$ mutation was included in the main challenge trial since an alanine substitution in the predicted N-glycosylation motif was viewed as a more secure substitution to ensure the absence of glycan at this site. Glycosylation at glutamine (Q), although exceptionally rare, has been documented (Vallieres-Douglass et al., 2010).

3.5 | Main challenge study

3.5.1 | Clinical signs and mortality

Clinical signs of fish may be difficult to assess since observations and interpretations are largely subjective. However, fish in the rSAV3 and rSAV3-Cap_{NLS} tanks were evaluated to display more sluggish behaviour as demonstrated by being easier to catch at sampling, while the rSAV3-$Cap_{NLS}E2_{319A}$ group appeared to be the least affected of the groups immunized with the mutated virus strains.

Mortality was observed in both the injected fish and cohabitants for rSAV3-$E2_{319A}$, rSAV3-Cap_{NLS} and rSAV3 (Figure 4). For these groups, cumulative mortality was 7.7%, 20.5% and 17.9% for groups injected with rSAV3-$E2_{319A}$, rSAV3-Cap_{NLS} and rSAV3, respectively. For cohabitants cumulative mortality was 5% for all three groups. No mortalities were observed in the rSAV3-$Cap_{NLS}E2_{319A}$, inactivated SAV3 vaccine (Figure 4) and PBS groups (not shown).

3.6 | Weight and length

The weight of the fish ranged from 40 to 50 g at the start of the challenge trial. At 2 wpi (not shown) and 4 wpi, there was no significant weight difference between groups injected with mutated, non-mutated inactivated virus or PBS (Figure 5). At 8 wpi, on the other hand, the weight in the control groups, that is inactivated SAV3 and PBS, was significantly higher ($p < .003$) compared with the groups injected with infectious virus (Figure 5). Here, the largest weight gain was observed in the PBS group (mean value 93 g) and lowest in the rSAV3 group (mean value 46 g). The trend observed at 8 wpi continued at 12 wpi. However, at this time point the rSAV3-$Cap_{NLS}E2_{319A}$ group had gained an average weight that was significantly higher compared with the groups injected with rSAV3 ($p < .007$), rSAV3-Cap_{NLS} ($p < .01$) and rSAV3-$E2_{319A}$ ($p < .4$; Figure 5). The difference between the mean weights of the inactivated virus group (92.0 g) to that of the $Cap_{NLS}E2_{319A}$ group (76.3 g) was not significant at this time point ($p = .08$). Weight of fish in the PBS and inactivated SAV3 groups at 8 wpi was significantly higher ($p < .001$ and $p = .004$, respectively) than groups injected with infectious virus. At 12 wpi, the weight of rSAV3-$Cap_{NLS}E2_{319A}$-injected group was significantly higher than for the other groups injected with the mutated virus, and

TABLE 5 Histopathological score of heart sampled from 6 fish from each group at 6 wpi.

PD score	$E2_{319A}$	Cap_{NLS}	$Cap_{NLS}E2_{319A}$	rSAV3	Inact.	PBS
0	0	0	0	0	3	2
1	1	1	0	1	3	3
2	0	3	5	2	0	1
3	4	1	1	2	0	0
4	1	1	0	1	0	0

The evaluation is scored subjectively in a semi-quantitatively manner (0=none, 1= very mild, 2= mild, 3= moderate, 4= severe).

no significant difference was observed between rSAV3$Cap_{NLS}E2_{319A}$ and the inactivated SAV3-injected group ($p > .08$).

At 4 wpi, the difference in length between the groups was not significant (Figure 5). At 8 and 12 wpi, fish injected with PBS and inactivated virus had a mean length significantly higher ($p < .004$ and $p < .002$, respectively) compared with the other groups (Figure 5). No significant differences ($p > .05$) in average lengths were observed for groups injected with infectious virus at 8 and 12 wpi (Figure 5). However, even if the difference in length was not significant at 12 wpi, the trend in length mirrored the mortality and weight scores (Figure 5).

3.7 | Viral RNA and antiviral immune response in heart

All fish immunized with mutated virus strains were positive for viral RNA in heart at 2 wpi (Figure 6). At 4 wpi, positive cohabitants were also observed, with all cohabitants from the rSAV3 group being positive, one of three in the rSAV3-$E2_{319A}$ group and two of three in groups rSAV3-Cap_{NLS} and rSAV3-$Cap_{NLS}E2_{319A}$. At 6 and 8 wpi, 54 out 56 (96.4%) of the fish immunized with mutated virus remained positive for virus in heart with increasing average Ct values. At 10 and 12 wpi, fish in groups immunized with mutated virus strains and their cohabitants were still positive for the vaccine strains. However, average Ct values had now increased to approximately 30 and 27 for injected and cohabitant fish, respectively.

No viral RNA was detected in fish injected with inactivated SAV3 prior to or after challenge.

We observed a difference in the number of RT-qPCR virus-positive fish in the cohabitant group of the rSAV3-$Cap_{NLS}E2_{319A}$ versus the cohabitants in the other groups injected with infectious virus. From the eighteen sampled cohabitant fish exposed to rSAV3-$Cap_{NLS}E2_{319A}$, 50% remained uninfected compared with 20.7%, 22.2% and 20.7% for rSAV3-$E2_{319A}$, rSAV3-Cap_{NLS} and rSAV3, respectively. For rSAV3-$E2_{319A}$ and rSAV3-$Cap_{NLS,}$ all cohabitants tested positive after 4 wpi.

Viral RNA obtained from heart at 12 wpi was amplified with PCR and Sanger-sequenced to determine whether the fish was infected with the injected clone or the challenge strain H20/03/2. All virus-positive fish at 12 wpi were infected with the injected strains except one rSAV3$Cap_{NLS}E2_{319A}$ cohabitant, which was infected with H20/03/2. The Ct value for this individual was 19.1, and weight was 109 g, compared with the group average of 66.5, which suggests this fish had never been infected with mutated virus. We did not find indication of double infections.

The innate antiviral response was assessed by measuring Mx and viperin mRNA levels. No significant differences in expression of Mx and viperin were observed between rSAV3-$E2_{319A}$, rSAV3-$Cap_{NLS}E2_{319A}$ and rSAV3. However, Mx expression in rSAV3-Cap_{NLS} fish was significantly lower at 2 wpi compared with the other groups injected with infectious virus. At 4 wpi, there were no significant differences in Mx and viperin expression levels between the groups infected with infectious virus. No significant differences in expression of Mx were found between the groups injected with inactivated virus and PBS. However, for viperin a significant difference ($p < .049$) was found between inactivated virus and PBS at 4 wpi. The viral RNA loads in heart correlate positively with expression of Mx and viperin (Figure 6).

3.8 | Histopathology

The pathology observed in the 36 hearts from the six different groups varied in score from none (0) to severe (4). The results are summarized in Table 5, and examples of scoring are shown in Figure 7.

The highest level of pathology, mainly severe lesions where more than 50% of the ventricle was affected with necrosis and inflammation, was observed in the hearts from fish in the rSAV3-$E2_{319A}$ group. The rSAV3 and rSAV3-Cap_{NLS} groups showed variation in pathology with mild, moderate and severe pathology. In the rSAV3-$Cap_{NLS}E2_{319A}$ group, the main findings were mild lesions observed in five of six fish. In the group injected with inactivated vaccine and in the non-exposed, PBS control group, only none to very mild degenerative lesions were observed. One individual in the PBS group revealed mild pathology.

4 | DISCUSSION

In recent years, significant progress has been made in the detailed mapping of alphavirus replication mechanisms, making targeted mutation attractive for attenuation purposes to developing effective vaccines (Dupuy et al., 2011; Kim, Atasheva, et al., 2011; Paessler & Weaver, 2009; Wang et al., 2007, 2008). The ideal level

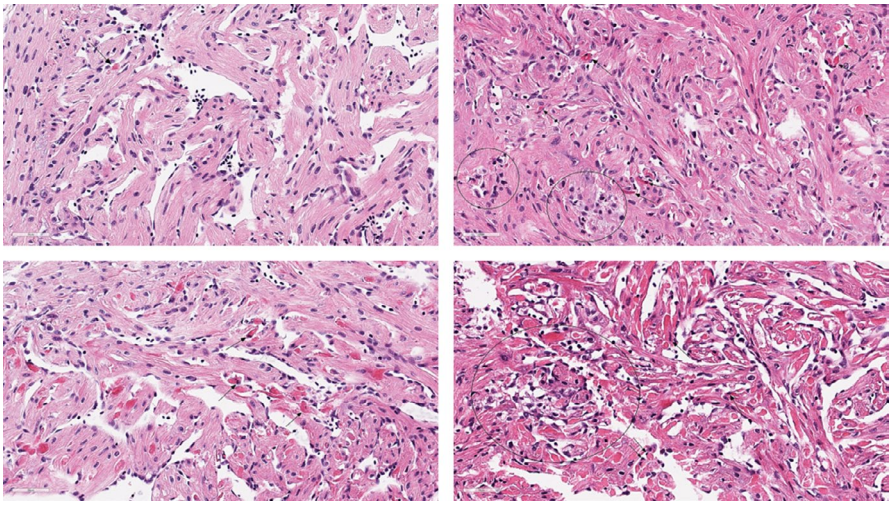

FIGURE 7 Examples of histopathological lesions in HE-stained fish heart sections, 6wpi: (a) Score 1: Most of the heart tissue appears normal; one necrotic myocardial fibre was observed in this area (arrow). (b) Score 2: More than 7 myocytes and less than 15% of the heart tissue was necrotic (arrows). There was a sparse infiltration of inflammatory cells (circles). (c) Score 3: More than 15% and less than 50% of the heart tissue was affected; in this individual, mainly necrotic myocardial fibres were observed (arrows). (d) Score 4: More than 50% of the heart tissue was affected; in this individual, both necrotic myocardial fibres (arrows) and inflammatory cell infiltration (circles) were present. Scale bar: 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]

of attenuation of a live-attenuated PD vaccine should be tuned such that the vaccine SAV strain is no longer virulent but still sufficiently stimulates the innate and specific immune responses. In general, a live-attenuated vaccine should give a controlled infection. The risk of unintentional infection of non-target species should be minimal, that is uncontrolled shedding of vaccine strains is undesired, and the possibility of reversion or compensational mutation causing reversion to virulence should be low. However, transmission of attenuated vaccine strains to cohabitants and migrating wild salmon is not necessarily a disadvantage, assuming the attenuated virus does not revert, due to the positive effect of immunity obtained in the farmed and wild populations. Although SAV conforms to many alphavirus characteristics, it does not group within either of the Old or New world phylogenetic clades. This suggests that an approach where a strategy for attenuation used for either Old or New world viruses or a combination of both could be a promising approach.

For New world viruses, a strategy to obtain attenuation has been to target the capsid protein due to its role as a key inhibitor of host cellular transcription (Atasheva et al., 2015; Garmashova, Gorchakov, et al., 2007). The alphavirus capsid protein is evolutionary conserved in the C terminus and highly variable in the N terminus. But all alphaviruses, including SAV, share a structural relationship through a helix I and a downstream positively charged region in the N-terminal part (Karlsen, Yousaf, et al., 2010; Perera et al., 2001). Studies on the New world virus VEEV demonstrated that transcriptional shutoff and development of cytopathic changes are regulated by the capsid protein (Aguilar et al., 2007, 2008; Garmashova, Atasheva, et al., 2007; Garmashova, Gorchakov, et al., 2007). Co-localization of the capsid protein with the nuclear pore complex and interference with nuclear–cytoplasmic trafficking is thought to be the cause of this cellular shutoff (Atasheva et al., 2008, 2010, 2015). Therefore, similar mutations in the NLS and NES region of the capsid were pursued to attenuate SAV.

For the Old world viruses Sindbis virus (SINV) and Ross River virus (RRV), mutating the N-glycosylation sites in the envelope glycoproteins E1 and E2 and thus eliminating glycan attachment at these sites cause attenuation as assessed both by in vitro and by in vivo experiments (Knight et al., 2009; Nelson et al., 2016; Smit et al., 2002).

In the present work, we tested three different mutated SAV3 infectious strains in the target species Atlantic salmon. The three strains contained mutations in either (a) the predicted N-glycosylation site in E2; (b) the NLS in the capsid protein; or (c) in both proteins. When the mutated viruses were injected into Atlantic salmon presmolts, infection was established for all three strains, and they were shed and infected cohabitant fish.

No mortality was observed in fish immunized with the double-mutated rSAV3-$Cap_{NLS}E2_{319A}$, while the highest mortality was observed for in the rSAV3-Cap_{NLS} group, being at the same level as the parental rSAV3 infectious clone. The trend observed in mortality was also mirrored in the weight, which was significantly higher at 12 wpi for the rSAV3-$Cap_{NLS}E2_{319A}$ than for the other groups. There were no significant differences in the length of fish in the different groups injected with the mutated strains or the parental strain. The body length of salmonid fish is expected to gradually increase over time although reduced growth in body length and even length shrinkage have been observed under harsh living conditions (Huusko et al., 2011).

Intramuscular injection of virus resulted in an acute infection followed by a persistent but declining detection of low levels of viral RNA in heart tissue throughout the 84 days of the experimental trial. SAV has earlier been detected as long as 140 and 190 days after

experimental infection (Andersen et al., 2007; Christie et al., 2007). RT-qPCR does not distinguish between unpackaged viral RNA, replication intermediates, defective virus particles or RNA originating from infectious virus. Therefore, we cannot conclude whether the detection of viral RNA during the later stages of the trial was due to active virus replication or from residual RNA. The lack of significant differences between the groups with regard to viral RNA loads in hearts suggests that the rates of replication were at similar levels for the mutated clones and the parental strain.

The measurement of Mx and viperin expression by RT-qPCR can serve as a method for detection of the innate antiviral response (Ellis, 2001; Robertsen et al., 1997). We found a positive correlation between viral RNA load and expression of Mx and viperin, which was not observed in fish injected with the inactivated SAV vaccine.

Severe pathology in heart caused by SAV has been observed 2–5 weeks after intraperitoneal injection (Andersen et al., 2010). In the present study, histopathology of hearts sampled at 6 wpi revealed pathology in the infected groups, which varied in score; however, there were no significant differences in histopathological score between the mutated strains and their parental strain. For rSAV3-$Cap_{NLS}E2_{319A}$, although not significant, the histopathology scores were lower on average compared with the other groups immunized with mutated virus strains.

The rSAV3-$E2_{319A}$ has earlier been found to be attenuated in cell culture, in vitro (Aksnes et al., 2020). However, in injected fish no significant differences in level of virus replication, or length of the fish were observed compared with the parental rSAV3 strain. However, the mutant virus did induce significant lower mortalities and the fish weighed slightly more on average at 12 weeks compared with rSAV3-infected fish. Therefore, conclusions made on level of attenuation based on measuring viral properties in cell culture systems should be performed with caution. Cell culture systems based on monocultures often represent good model systems, but they are rarely ideal substitutes for in vivo infection studies in the natural host.

The infectious rSAV3 clone was originally designed to be identical in sequence to the wild-type H20/03/2 isolate, and rSAV3 has been shown to produce viral titres that are comparable to H20/03/2 in cell culture (Aksnes et al., 2020; Karlsen, Villoing, et al., 2010). The full-length sequencing of prSAV3 and H20/03/2 revealed twelve silent and four non-silent nucleotide differences, where the four amino acids in prSAV3 that differ from H20/03/2 have all been reported in other SAV3 field isolates. In fact, seven of the twelve nucleotide differences were unique for H20/03/2 (Table S1). This suggests that the recovered SAV3 from the cDNA clone, that is rSAV3, is more similar to field isolates than to the cell culture adapted variant of H20/03/2 used in our laboratory. This variant has, as previously mentioned, undergone an unknown number of passages in cell culture. Cell culture adaptation has been observed for other alphaviruses (Sammels et al., 1995; Weaver et al., 1999), as well as for SAV3 (Karlsen et al., 2006). The H20/03/2 strain was therefore considered not to be a proper control strain in the present study, as it differed substantially in sequence from prSAV3 and the mutated infectious strains derived thereof. The observed differences in mortality or disease characteristics might therefore have originated from other pre-existing nucleotide differences, had the H20/03/2 strain been used as control. The viral quasispecies composition existing after multiple passages in cell culture may also influence infection and can be a critical component of viral fitness (Vignuzzi et al., 2006). Viral quasispecies during alphavirus infection, including SAV, are well documented and studied (Petterson et al., 2013; Stapleford et al., 2016).

A major finding from the experimental infection trial was that the introduced mutations were still present and that the mutant viruses all caused infection that persisted throughout the course of the trial. Also, the inability to detect the challenge strain, that is H20/03/02, suggests that the challenge strain did not infect the immunized fish. The observation that the introduced mutations did not revert was an important finding. Live-attenuated vaccines have an inherent risk of reversion to virulence. An important characteristic of alphaviruses is their resilience and rapid evolution, resulting in accumulation of mutations giving adaptations of more efficiently replicating phenotypes in vitro or higher virulence in vivo (Ciota & Kramer, 2010). An example is a live, attenuated vaccine developed against VEEV, TC-83, that causes adverse events in clinical trials due to reversion of some mutations (Paessler & Weaver, 2009).

The high mutation rates of alphaviruses may result in rapid evolution and possible selection of efficiently transmitting phenotypes in a setting with high densities of susceptible hosts such as salmon farming. Furthermore, an increase in virulence may also be due to compensational mutations at other sites in the viral genome and not an actual reversal of the attenuating mutations. In a study on a strain of VEEV, which lacked a conserved 51 nucleotide sequence in nsP1, a rapid accumulation of compensatory mutations in nsP2 and nsP3 resulted in accelerated virus growth, which was observed after few rounds of infection (Michel et al., 2007).

There are currently no available live-attenuated vaccines developed against diseases caused by salmonid alphavirus. Development of alphavirus variants that are highly attenuated by introducing changes in the genomic RNA packaging signals has been suggested (Frolova et al., 1997). In VEEV, numerous synonymous mutations in the RNA packaging signal (PS), which did not change the nsP1-specific amino acid sequence but modified the nucleotide sequence and secondary structure of the VEEV PS, strongly affected the release of infectious, genome-containing virions and infectious titre (Kim, Firth, et al., 2011). It has been suggested that SAV has an important packaging signal located in a region of nsP2 and that mutations located near this region can impact replication (Moriette et al., 2006).

The infectious strains used in this work were not sufficiently attenuated in vivo and are therefore not good candidates for vaccination purposes without further modifications. However, the strain rSAV3$Cap_{NLS}E2_{319A}$, carrying mutations in both the capsid and E2, induced lower mortality and higher weight gain in the fish compared with the parental rSAV3. The introduction of multiple targeted mutations in rSAV3 may therefore represent an attenuation strategy that also reduces the risk of reversion for a potential live vaccine candidate.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funder had no role in the design of the study: in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

AUTHOR CONTRIBUTIONS

I. A. S. V. and E. R. contributed to conceptualization; S. B. and I. A. contributed to methodology; I. A and T. M. contributed to software; S. B. made validation; I. A., T. M., C. P.Å, S. V. and E. R. made formal analysis; I. A. and E. R. made investigation; E. R. contributed to resources; T. M. made data curation; I. A. C. P.Å. and E. R. involved in writing—original draft preparation; E. R., T. M., I. A., S. V. and C. P.Å in writing—review and editing; E. R. administrated the project; E. R. acquired the funding. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

Yes, the research data acquired in this work are open and available. Norwegian University of Life Sciences adheres to the FAIR principles, Findable, Accessible, Interoperable and Reusable.

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REFERENCES

Aguilar, P. V., Leung, L. W., Wang, E., Weaver, S. C., & Basler, C. F. (2008). A five-amino-acid deletion of the eastern equine encephalitis virus capsid protein attenuates replication in mammalian systems but not in mosquito cells. *Journal of Virology*, *82*, 6972–6983. https://doi.org/10.1128/JVI.01283-07

Aguilar, P. V., Weaver, S. C., & Basler, C. F. (2007). Capsid protein of eastern equine encephalitis virus inhibits host cell gene expression. *Journal of Virology*, *81*, 3866–3876.

Akhrymuk, I., Kulemzin, S. V., & Frolova, E. I. (2012). Evasion of the innate immune response: The old world alphavirus nsP2 protein induces rapid degradation of Rpb1, a catalytic subunit of RNA polymerase II. *Journal of Virology*, *86*, 7180–7191. https://doi.org/10.1128/JVI.00541-12

Aksnes, I., Markussen, T., Braaen, S., & Rimstad, E. (2020). Mutation of N-glycosylation sites in salmonid alphavirus (SAV) envelope proteins attenuate the virus in cell culture. *Viruses*, *12*, 1071.

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.). *Archives of Virology*, *152*, 1871–1883.

Andersen, L., Hodneland, K., & Nylund, A. (2010). No influence of oxygen levels on pathogenesis and virus shedding in Salmonid alphavirus (SAV)-challenged Atlantic salmon (*Salmo salar* L.). *Virology Journal*, *7*, 198., https://doi.org/10.1186/1743-422X-7-198

Atasheva, S., Fish, A., Fornerod, M., & Frolova, E. I. (2010). Venezuelan equine encephalitis virus capsid protein forms a tetrameric complex with CRM1 and importin alpha/beta that obstructs nuclear pore complex function. *Journal of Virology*, *84*, 4158–4171.

Atasheva, S., Garmashova, N., Frolov, I., & Frolova, E. (2008). Venezuelan equine encephalitis virus capsid protein inhibits nuclear import in mammalian but not in mosquito cells. *Journal of Virology*, *82*, 4028–4041.

Atasheva, S., Kim, D. Y., Frolova, E. I., & Frolov, I. (2015). Venezuelan equine encephalitis virus variants lacking transcription inhibitory functions demonstrate highly attenuated phenotype. *Journal of Virology*, *89*, 71–82.

Christie, K. E., Graham, D. A., McLoughlin, M. F., Villoing, S., Todd, D., & Knappskog, D. (2007). Experimental infection of Atlantic salmon Salmo salar pre-smolts by i.p. injection with new Irish and Norwegian salmonid alphavirus (SAV) isolates: A comparative study. *Diseases of Aquatic Organisms*, *75*, 13–22. https://doi.org/10.3354/dao075013

Ciota, A. T., & Kramer, L. D. (2010). Insights into arbovirus evolution and adaptation from experimental studies. *Viruses*, *2*, 2594–2617.

Cruz, C. C., Suthar, M. S., Montgomery, S. A., Shabman, R., Simmons, J., Johnston, R. E., Morrison, T. E., & Heise, M. T. (2010). Modulation of type I IFN induction by a virulence determinant within the alphavirus nsP1 protein. *Virology*, *399*, 1–10.

Dalmo, R. A. (2018). DNA vaccines for fish: Review and perspectives on correlates of protection. *Journal of Fish Diseases*, *41*, 1–9.

Dupuy, L. C., Richards, M. J., Ellefsen, B., Chau, L., Luxembourg, A., Hannaman, D., Livingston, B. D., & Schmaljohn, C. S. (2011). A DNA vaccine for venezuelan equine encephalitis virus delivered by intramuscular electroporation elicits high levels of neutralizing antibodies in multiple animal models and provides protective immunity to mice and nonhuman primates. *Clinical and Vaccine Immunology*, *18*, 707–716. https://doi.org/10.1128/CVI.00030-11

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Developmental and Comparative Immunology*, *25*, 827–839.

Favre, D., Studer, E., & Michel, M. R. (1994). 2 Nucleolar targeting signals present in the N-terminal part of Semliki forest virus capsid protein. *Archives of Virology*, *137*, 149–155.

Frolova, E., Frolov, I., & Schlesinger, S. (1997). Packaging signals in alphaviruses. *Journal of Virology*, *71*, 248–258.

Fros, J. J., van der Maten, E., Vlak, J. M., & Pijlman, G. P. (2013). The C-terminal domain of Chikungunya virus nsP2 independently governs viral rna replication, cytopathicity, and inhibition of interferon signaling. *Journal of Virology*, *87*, 10394–10400. https://doi.org/10.1128/JVI.00884-13

Galbraith, S. E., Sheahan, B. J., & Atkins, G. J. (2006). Deletions in the hypervariable domain of the nsP3 gene attenuate Semliki Forest virus virulence. *Journal of General Virology*, *87*, 937–947.

Garmashova, N., Atasheva, S., Kang, W., Weaver, S. C., Frolova, E., & Frolov, I. (2007). Analysis of Venezuelan equine encephalitis virus capsid protein function in the inhibition of cellular transcription. *Journal of Virology*, *81*, 13552–13565.

Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E., & Frolov, I. (2007). The old world and new world alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *Journal of Virology*, *81*, 2472–2484.

Hodneland, K., & Endresen, C. (2006). Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan). *Journal of Virological Methods*, *131*, 184–192.

Huusko, A., Maki-Petays, A., Stickler, M., & Mykra, H. (2011). Fish can shrink under harsh living conditions. *Functional Ecology*, *25*, 628–633.

Jalanko, A., & Soderlund, H. (1985). THE Repeated regions of Semliki forest virus defective-inferfering RNA interferes with the encapsidation process of the standard virus. *Virology*, *141*, 257–266.

Jensen, B. B., Kristoffersen, A. B., Myr, C., & Brun, E. (2012). Cohort study of effect of vaccination on pancreas disease in Norwegian salmon aquaculture. *Diseases of Aquatic Organisms*, *102*, 23–U33.

Johnson, B. J. B., Kinney, R. M., Kost, C. L., & Trent, D. W. (1986). Molecular determinants of alphavirus neurovirulence - nucleotide and deduced protein-sequence changes during attenuation of Venezuelan equine encephalitis-virus. *Journal of General Virology*, *67*, 1951–1960.

Jones, D. T. (1999). Protein secondary structure prediction based on position-specific scoring matrices. *Journal of Molecular Biology*, *292*, 195–202.

Jupille, H. J., Oko, L., Stoermer, K. A., Heise, M. T., Mahalingam, S., Gunn, B. M., & Morrison, T. E. (2011). Mutations in nsP1 and PE2 are critical determinants of Ross River virus-induced musculoskeletal inflammatory disease in a mouse model. *Virology*, *410*, 216–227. https://doi.org/10.1016/j.virol.2010.11.012

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

Karlsen, M., Tingbo, T., Solbakk, I. T., Evensen, O., Furevik, A., & Aas-Eng, A. (2012). Efficacy and safety of an inactivated vaccine against Salmonid alphavirus (family Togaviridae). *Vaccine*, *30*, 5688–5694.

Karlsen, M., Villoing, S., Ottem, K. F., Rimstad, E., & Nylund, A. (2010). Development of infectious cDNA clones of Salmonid alphavirus subtype 3. *BMC Research Notes*, *3*, 241.

Karlsen, M., Yousaf, M. N., Villoing, S., Nylund, A., & Rimstad, E. (2010). The amino terminus of the salmonid alphavirus capsid protein determines subcellular localization and inhibits cellular proliferation. *Archives of Virology*, *155*, 1281–1293.

Kim, D. Y., Atasheva, S., Foy, N. J., Wang, E. Y., Frolova, E. I., Weaver, S., & Frolov, I. (2011). Design of chimeric alphaviruses with a programmed, attenuated, cell type-restricted phenotype. *Journal of Virology*, *85*, 4363–4376.

Kim, D. Y., Firth, A. E., Atasheva, S., Frolova, E. I., & Frolov, I. (2011). Conservation of a packaging signal and the viral genome RNA packaging mechanism in alphavirus evolution. *Journal of Virology*, *85*, 8022–8036.

Knight, R. L., Schultz, K. L. W., Kent, R. J., Venkatesan, M., & Griffin, D. E. (2009). Role of N-linked glycosylation for Sindbis virus infection and replication in vertebrate and invertebrate systems. *Journal of Virology*, *83*, 5640–5647.

Kongtorp, R. T., Stene, A., Andreassen, P. A., Aspehaug, V., Graham, D. A., Lyngstad, T. M., Olsen, A. B., Olsen, R. S., Sandberg, M., & Santi, N. (2010). Lack of evidence for vertical transmission of SAV 3 using gametes of Atlantic salmon, *Salmo salar* L., exposed by natural and experimental routes. *Journal of Fish Diseases*, *33*, 879–888.

la Cour, T., Kiemer, L., Mølgaard, A., Gupta, R., Skriver, K., & Brunak, S. (2004). Analysis and prediction of leucine-rich nuclear export signals. *Protein Engineering Design and Selection*, *17*, 527–536.

Levinson, R. S., Strauss, J. H., & Strauss, E. G. (1990). Complete sequence of the genomic RNA of O'nyong-nyong virus and its use in the construction of alphavirus phylogenetic trees. *Virology*, *175*, 110–123. https://doi.org/10.1016/0042-6822(90)90191-S

McBeath, A. J. A., Snow, M., Secombes, C. J., Ellis, A. E., & Collet, B. (2007). Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (*Salmo salar*) following infection with infectious pancreatic necrosis virus and infectious salmon anaemia virus. *Fish & Shellfish Immunology*, *22*, 230–241. https://doi.org/10.1016/j.fsi.2006.05.004

McCleary, S., Giltrap, M., Henshilwood, K., & Ruane, N. M. (2014). Detection of salmonid alphavirus RNA in Celtic and Irish Sea flatfish. *Diseases of Aquatic Organisms*, *109*, 1–7.

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

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. *Diseases of Aquatic Organisms*, *72*, 125–133.

McLoughlin, M. F., Nelson, R. N., McCormick, J. I., Rowley, H. M., & Bryson, D. B. (2002). Clinical and histopathological features of naturally occurring pancreas disease in farmed Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases*, *25*, 33–43.

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. *Journal of Virology*, *87*, 6027–6030. https://doi.org/10.1128/JVI.03501-12

Michel, G., Petrakova, O., Atasheva, S., & Frolov, I. (2007). Adaptation of Venezuelan equine encephalitis virus lacking 51-nt conserved sequence element to replication in mammalian and mosquito cells. *Virology*, *362*, 475–487.

Michel, M. R., Elgizoli, M., Dai, Y., Jakob, R., Koblet, H., & Arrigo, A. P. (1990). Karyophilic properties of Semliki forest virus nucleocapsid protein. *Journal of Virology*, *64*, 5123–5131.

Minor, P. D. (2015). Live attenuated vaccines: Historical successes and current challenges. *Virology*, *479*, 379–392.

Mitchell, C., Deandraderozental, A. F., Soutopadron, T., & Carvalho, M. D. D. (1997). Identification of mayaro virus nucleocapsid protein in nucleus of *Aedes albopictus* cells. *Virus Research*, *47*, 67–77.

Moriette, C., Leberre, M., Lamoureux, A., Lai, T. L., & Bremont, M. (2006). Recovery of a recombinant salmonid alphavirus fully attenuated and protective for rainbow trout. *Journal of Virology*, *80*, 4088–4098.

Nelson, M. A., Herrero, L. J., Jeffery, J. A. L., Hoehn, M., Rudd, P. A., Supramaniam, A., Kay, B. H., Ryan, P. A., & Mahalingam, S. (2016). Role of envelope N-linked glycosylation in Ross River virus virulence and transmission. *Journal of General Virology*, *97*, 1094–1106.

Paessler, S., & Weaver, S. C. (2009). Vaccines for venezuelan equine encephalitis. *Vaccine*, *27*, D80–D85.

Perera, R., Owen, K. E., Tellinghuisen, T. L., Gorbalenya, A. E., & Kuhn, R. J. (2001). Alphavirus nucleocapsid protein contains a putative coiled coil α-helix important for core assembly. *Journal of Virology*, *75*, 1–10.

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

Robertsen, B., Trobridge, G., & Leong, J. A. (1997). Molecular cloning of double-stranded RNA inducible Mx genes from Atlantic salmon (*Salmo salar* L.). *Developmental and Comparative Immunology*, *21*, 397–412.

Sammels, L. M., Coelen, R. J., Lindsay, M. D., & Mackenzie, J. S. (1995). Geographic-distribution and evolution of Ross river virus in Australia and the Pacific Islands. *Virology*, *212*, 20–29.

Simmons, J. D., Wollish, A. C., & Heise, M. T. (2010). A determinant of sindbis virus neurovirulence enables efficient disruption of Jak/STAT signaling. *Journal of Virology*, *84*, 11429–11439.

Skjold, P., Sommerset, I., Frost, P., & Villoing, S. (2016). Vaccination against pancreas disease in Atlantic salmon, *Salmo salar* L., reduces shedding of salmonid alphavirus. *Veterinary Research*, *47*.

Smit, J. M., Mukhopadhyay, S., Kuhn, R. J., & Wilschut, J. (2002). The role of N-linked glycosylation of Sindbis virus glycoproteins E2 and E1 in viral infectivity and membrane fusion activity. Mutational Analysis of Receptor Interaction and Membrane Fusion Activity of Sindbis Virus, 107.

Stapleford, K. A., Moratorio, G., Henningsson, R., Chen, R., Matheus, S., Enfissi, A., Weissglas-Volkov, D., Isakov, O., Blanc, H., & Mounce, B. C. J. P. N. T. D. (2016). *Whole-genome Sequencing Analysis from the Chikungunya Virus Caribbean Outbreak Reveals Novel Evolutionary Genomic Elements*., *10*, e0004402.

Strom, A. C., & Weis, K. (2001). Importin-beta-like nuclear transport receptors. *Genome Biology*, *2*(6), reviews3008.1–reviews3008.9.

Valliere-Douglass, J. F., Eakin, C. M., Wallace, A., Ketchem, R. R., Wang, W., Treuheit, M. J., & Balland, A. (2010). Glutamine-linked and non-consensus asparagine-linked oligosaccharides present in human recombinant antibodies define novel protein glycosylation motifs. *Journal of Biological Chemistry*, *285*, 16012–16022.

Vignuzzi, M., Stone, J. K., Arnold, J. J., Cameron, C. E., & Andino, R. (2006). Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature*, *439*, 344–348.

Villoing, S., Bearzotti, M., Chilmonczyk, S., Castric, J., & Bremont, M. (2000). Rainbow trout sleeping disease virus is an atypical alphavirus. *Journal of Virology*, *74*, 173–183.

Wang, E., Petrakova, O., Adams, A. P., Aguilar, P. V., Kang, W. L., Paessler, S., Volk, S. M., Frolov, I., & Weaver, S. C. (2007). Chimeric sindbis/eastern equine encephalitis vaccine candidates are highly attenuated and immunogenic in mice. *Vaccine*, *25*, 7573–7581.

Wang, E., Volkova, E., Adams, A. P., Forrestera, N., Xiao, S. Y., Frolov, I., & Weaver, S. C. (2008). Chimeric alphavirus vaccine candidates for chikungunya. *Vaccine*, *26*, 5030–5039.

Weaver, S. C., Brault, A. C., Kang, W. L., & Holland, J. J. (1999). Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells. *Journal of Virology*, *73*, 4316–4326.

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. *Veterinary Research*, *43*, 78.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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