**INTRODUCTION**

Salmonid 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).
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 (Méroué 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 H10P3, H10P11 and H10P14, 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 resulted 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 (H10P3), a late passage on CHSE (H10P11) and an AGK-transferred passage (H10P14) were subjected to plaque purification and

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**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 (H10P3) and 11th (H10P11) passage on CHSE and the passage resulting from nine passages on CHSE followed by five passages on AGK (H10P14) 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 H10P14 and H10P3 at m.o.i. 10. (a) CHSE cells infected with H10P14 at 4 days p.i. Shrinkage (*) and swelling (arrowhead) of single cells is indicated. (b) CHSE cells infected with H10P14 at 14 days p.i. (c) CHSE cells infected with H10P3 at 14 days p.i. (d) CHSE cells non-infected at 14 days p.i. (e) AGK cells infected with H10P14 at 4 days p.i. (f) AGK cells infected with H10P14 at 14 days p.i. (g) AGK cells infected with H10P3 at 14 days p.i. (h) AGK cells non-infected at 14 days p.i.
subsequent characterization, CPE induced by H10P3, H10P11 and H10P14 isolates in CHSE and AGK cells was assessed morphologically by phase-contrast microscopy from 0 h to 14 days post-infection (p.i.). H10P14 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 H10P3. CPE developed from H10P11 did not differ greatly from that developed from H10P3. 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 H10P14 were lysed by 7 days p.i. with a few cells surviving in all wells at 14 days p.i. (Fig. 2b). For H10P3 and H10P11, 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 H10P14 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 H10P14 and increased with time p.i. resulting in almost complete lysis (Fig. 2f), while H10P3 and H10P11 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. H10P14 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). H10P3 resulted in a loss of viability compared with controls by 6 days p.i. (also significantly different from H10P14) in CHSE cells. In AGK cells there was a significant difference between the two strains (H10P14 and H10P3) 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 H10P14 (Fig. 3b). Infection using H10P11 resulted in an intermediate (or similar to H10P3) effect on cell viability compared with H10P3 and H10P14 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 H10P11 and H10P14 at m.o.i. 10. The results in CHSE indicated that strain H10P14 has a higher replication in CHSE compared with H10P11 as the levels of viral load increased significantly up to day 4 for H10P14, while for H10P11 there was no significant increase after day 2. Although, the data indicate that H10P14 reached the highest level of viral RNA, the differences from H10P11 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 H10P3, H10P11 and H10P14, 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 H10P14 to H10P3 and H10P11, there was a significantly higher viral load at days 6, 8 and 10.
Viral replication of the three SAV3-H10 passage isolates measured by real-time PCR and given as mean Cp-values with SEM. (a) H10P11 and H10P14 in CHSE cell line. (b) H10P3, H10P11 and H10P14 in AGK cell line. Relevant P values are indicated with brackets (**P<0.001, *P<0.01, +P<0.05). a, H10P14 P value indication to H10P11 and H10P3 on the equivalent days p.i. (AGK 0 days p.i., only significant difference between H10P3 and H10P14); b, only one well.

(Fig. 4b). H10P14 replicates to higher copy numbers than the two other strains (25-fold higher than H10P11 at day 10), which corresponds well with cell viability data (Fig. 3b). Further, there was no significant difference between H10P3 and H10P11 (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 H10P14 was derived, H10P14 resulted in higher viral titres compared with the CHSE-derived H10P11 and H10P3 (Fig. 4b). H10P3 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 H10P3 and in a parallel tank a similar infection was performed using H10P14 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. H10P3 resulted in a significantly higher viral load in heart tissue than did H10P14 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 H10P3 and H10P14 was due to a change in cell tropism. The histology scores for pancreas corresponded with viral load in heart. H10P3 infection resulted in significantly higher scores than H10P14 at weeks 2 and 5 (Fig. 5b), showing that H10P3 has higher virulence and replication capacity compared with reduction for H10P14 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 H10P3, H10P11 and H10P14. 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 H10P14 isolate, which was present in the CHSE-grown H10P2 and AGK-grown H10P12 supernatants; in AGK-grown H10P13, 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 H10P14 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
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 H10P3 and H10P11 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 H10P14 contained a 3 nt deletion at positions of 66 to 68 as compared with H10P3 and H10P11 (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 (H10P3) and a later (H10P11) passage isolate obtained after three and 11 passages on CHSE-214 were characterized by in vitro studies together with an isolate (H10P14) 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 (H10P14) 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 H10P3 and H10P11 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. H10P14 had unique amino acids at two of the four positions and a 3 nt deletion in the 3'UTR compared with H10P3 and H10P11 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 protein have been found to vary between isolates from the field (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

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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 H10P3 and H10P14, 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.
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

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<tr>
<th>Plaque-purified isolate</th>
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<tr>
<td>H10P3</td>
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<tr>
<td>H10P11</td>
<td>G L Q N</td>
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<tr>
<td>H10P14</td>
<td>G F K D</td>
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<td>E1 81 E1 441 E2 133 NsP1 183</td>
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<td>G L (P³) Q D</td>
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<td>H10P10-P13 in AGK</td>
<td>G L/F³ Q D</td>
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<th>Plaque-purified isolate</th>
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<td>H10P11 isolate 2-7</td>
<td>G L Q D</td>
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<tr>
<td>H10P14 isolate 2-11</td>
<td>G F/L³ K D</td>
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*³P10/P11 L only, P12 F only, P13 mix of L and F
³²L in two of 10 isolates

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).
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 H10P3 and H10P11 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 H10P3 against H10P11 indicate a slightly higher virulence for H10P11 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 H10P11 versus H10P14 in CHSE and similarly H10P3 versus H10P14 in AGK. Although H10P14 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 (H10P14) 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 H10P14 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 H10P14 compared with that of H10P3 and H10P11 (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 (H10P14) 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 H10P3 and H10P11, 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 isolates (H10P14) 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 earlier findings.
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/composition 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 H10P14 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 Groupers 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). 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 passed 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 passed an additional five times (p9 to p14), also with harvest at 7–10 days post-inoculation. At passage, virus supernatant was clarified by low speed centrifugation before harvest at 7–10 days post-inoculation. At passage, virus supernatant was harvested by one freeze–thaw cycle unless full CPE was observed. From passage five, each passage isolate was plaque-purified before full-length genome determination of the residues/combination of residues referring to as H10P14, one later passage (11th on CHSE) referred to as H10P11 and one from a passage obtained from nine passages on CHSE followed by five passages on AGK referred to as H10P14, 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 (10⁻⁷ to 10⁻¹) of cell culture supernatants from passages H10P3, H10P11 and H10P14 and inoculated 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. Plates 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 cm² flasks and subsequently passed three times using conditions as described earlier for virus-inoculated cells. When CPE was evident, cells and supernatant were frozen and thawed before further passing 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 TCID₅₀ by the method of Kärber (1931), prior to cell infection assays and fish challenge (described below). Titration after plaque purification showed that H10P14 resulted in the highest titre (10⁸.8 TCID₅₀ ml⁻¹) and H10P11 (10⁷ TCID₅₀ ml⁻¹) and H10P3 the lowest (10⁶ TCID₅₀ 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 H10P3, H10P11 and H10P14 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 CellTitre 96 AQueous 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 CellTitre 96 AQueous 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 H10P11 and H10P14 at m.o.i. 10 in triplicate on 24-well plates of CHSE and AGK cell lines. H10P3 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 H10P11 and H10P14, and at 0, 2, 4, 6 (one parallel only) and 8 days p.i. for H10P3. 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\(^{P4}\), respectively, by
intramuscular injection of 100 \(\mu\)l supernatant from infected cell cul-
tures (10\(^{6.5}\)–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
tank control. The fish were sedated with approximately 17 mg l\(^{-1}\)
AQUI-S and euthanized by a sharp blow to the head before heart and head	
tissue were sampled and preserved in RNAlater (Invitrogen). Samples were stored at –20 °C until RNA	
to extraction was performed. Parallel samples from heart, pancreas and
tissues and skeletal muscle were submerged in 10% phosphate-buffered formalin
and processed for paraffin-embedding and sectioning. Tissue speci-
tified 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 RNasey Fibrous tissue mini kit and RNasey mini kit (Qiagen), respectively,
according to the kit protocols. The tissue was homogenized in RLT	
buffer with \(\beta\)-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 \(\mu\)g 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
\(m\) l 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\(^{P3}\) genome sequence as the cDNA template (Guo et al., 2014; SAV3-H10 GenBank accession no. JQ799939.1) and H10\(^{P3}\) and H10\(^{P4}\) 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-415) 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’ EcoRI and 3’ AflII/NotI restriction sites and 5’ EcoRI/AflII and 3’ NotI, 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 EcoRI and NotI 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 AflII and NotI 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 AflII/NotI sites, resulting in a plasmid with a full-length genome of the virus. Full-length sequences of H10\(^{P3}\), H10\(^{P11}\) and H10\(^{P4}\) 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\(^{P4}\) 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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