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Concentration and detection of salmonid alphavirus in seawater during a post-smolt salmon (*Salmo salar*) cohabitant challenge

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ABSTRACT: Currently, the prevalence of salmonid alphavirus (SAV) in Norwegian Atlantic salmon farms is largely surveyed via sacrificing fish and sampling of organ tissue on a monthly basis. However, a more cost-efficient, straightforward, rapid, reliable, reproducible and animal welfare friendly method based on the detection of SAV in water could be considered as an alternative method. In the present study, such a method was developed and optimized through a 6 wk cohabitant challenge trial, using post-smolt Atlantic salmon *Salmo salar* L challenged with high or low doses of SAV subtype 3 (SAV3). Tank water and tissue samples from cohabitant fish were collected at 16 time points. SAV3 was concentrated from the water by filtration, using either electronegative or electropositive membrane filters, which were subsequently rinsed with one of 4 different buffer solutions. SAV3 was detected first in tank water (7 d post-challenge, DPC), and later in cohabitant fish organ tissue samples (12 DPC). The electronegative filter (MF-Millipore™) and rinsing with NucliSENS® easyMAG® Lysis Buffer presented the best SAV3 recovery. A significant positive correlation was found between SAV3 in the tank water concentrates and the mid-kidney samples. Based on these results, detection of SAV3 in filtrated seawater is believed to have the potential to serve as an alternative method for surveillance of SAV in Atlantic salmon farms.

KEY WORDS: SAV detection · SAV concentration · Water filtration · Surveillance · RT-qPCR · Membrane filters · Salmonid alphavirus · Pancreas disease

1. INTRODUCTION

Pancreas disease (PD) is considered to be one of the most serious virus diseases in sea-farmed salmonids (Sommerset et al. 2020), resulting in significant economic consequences (Jansen et al. 2015). PD was discovered for the first time in 1976 in Scotland (Munro et al. 1984) and first reported in Norway more than a decade later (Poppe et al. 1989). The aetiological agent for PD was first reported in 1995 (Nelson et al. 1995) and is described as an enveloped, positive-sense single-stranded RNA virus (+ssRNA) (Deperasińska et al. 2018), formally named *Salmonid alphavirus* (SAV) (Weston et al. 2002).

SAV belongs to the genus *Alphavirus* in the family *Togaviridae* (Nelson et al. 1995, Weston et al. 2002) and, based on phylogenetic analysis of the partial E2 gene and nsP3 gene in SAV from farmed Atlantic salmon *Salmo salar* L. and rainbow trout *Oncorhynchus mykiss*, this virus can be divided into 6 subtypes (i.e. SAV1–SAV6) (Fringuelli et al. 2008). In Norway, there are currently 2 known subtypes (SAV2 and SAV3), and they are primarily distributed into 2 different PD endemic zones (Hjortaas et al. 2016). SAV3

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has so far only been found in Norway, in aquaculture of rainbow trout and Atlantic salmon (Hodneland et al. 2005).

Various experimental challenge studies have shown that SAV spreads via horizontal transmission (McLoughlin et al. 1996, Andersen et al. 2010, Xu et al. 2012, Graham et al. 2012, Jarungsriapisit et al. 2016a,b, 2020). In some of these studies it was also possible to detect SAV directly from seawater (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020). However, the current methods for detection of SAV and diagnosis of PD involve sampling of fish, followed by histopathology and quantitative real-time PCR (RT-qPCR). In 2007, PD became a notifiable fish disease (list 3) in Norway. National regulation was introduced in order to reduce the consequences of PD in a defined PD zone and to prevent further spread of SAV. Revision of the regulations in 2017 introduced different control zones for SAV2 and SAV3, as well as a surveillance zone.

The surveillance programme requires monthly sampling for PCR analysis of Atlantic salmon, trout, rainbow trout and char from all marine operative fish farming sites without current status as SAV-infected (Lovdata 2017). This results in the sacrifice of thousands of fish every year and is a time-consuming and resource-demanding approach that relies on analysing a relatively small number of fish that should represent the whole population on a site. Hence, in the beginning of an outbreak, when few individuals are infected, the likelihood of identifying the infected fish is small. A water sample that represents the environment for the total population in the farm may therefore serve a possible alternative due to fish shedding SAV into the water. Monitoring of the aquatic environment for harmful pathogens by filtration of water has been found to be successful, as reported for the oomycete *Aphanomyces astaci* which causes the crayfish plague (Strand et al. 2019, Rusch et al. 2020) and for the fish ectoparasite *Gyrodactylus salaris* (Rusch et al. 2018), and constitutes an animal welfare friendly method, with a potential for improved management strategies.

It has been reported that filtering water through charged membrane filters is a useful tool for concentrating viruses from water (Cashdollar & Wymer 2013). This simple and efficient technique is known as virus adsorption–elution (viradel), and has been described in several studies (Wallis et al. 1972, Farrah et al. 1976, Goyal & Gerba 1983), including SAV challenge trials (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020). Recently, we carried out an *in vitro* study involving filtration of artificial and natural seawater that was spiked with SAV3 (Weli et al. 2021). Virus in water samples was concentrated by adsorption to either electronegative or electropositive membrane filters, followed by rinsing of filters with one of 4 different buffer solutions, and quantifying using RT-qPCR and reverse-transcription droplet digital PCR (RT-ddPCR).

In the present study, our aim was to evaluate the potential of these 5 methods in detecting SAV3 directly from seawater holding infected post-smolt Atlantic salmon. Therefore, a 6 wk cohabitant challenge study was performed, with sampling of tank water and fish tissue at 16 time points. An efficient concentration method (CM) for SAV in water may serve as a cost-efficient, straightforward, rapid, reliable and reproducible process for detection of SAV in Atlantic salmon farms compared to screening of fish. Such a method may result in earlier identification of infection in a fish population and prevention of further virus spread. This method may also be developed for other waterborne pathogenic viruses, thus enabling earlier disease control measures and improved virological safety of the water environment.

2. MATERIALS AND METHODS

2.1. Experimental design

A cohabitant challenge trial was carried out in 3 separate tanks for a duration of 38 d. The number of shedder fish and cohabitant fish in each tank was 15 and 55, respectively. During the challenge period, sampling of both tank water and cohabitant fish was performed at 16 time points (see Fig. 1).

2.2. Experimental fish

The Atlantic salmon used in this trial arrived as eyed eggs when they were approximately 370 degree-days and were reared at the fish facility at the Industrial and Aquatic Laboratory (ILAB, Bergen High Technology Centre). In total, 210 post-smolts of Atlantic salmon of strain Stofnfiskur Iceland (SF Optimal), with an average weight of 110.9 g, were used in the challenge, which took place approximately 1 yr after hatching. The fish were unvaccinated and pre-screened (at 5 and 15 g of size) and tested negative for SAV, infectious salmon anaemia virus (ISAV), infectious pancreatic necrosis virus (IPNV), piscine myocarditis virus (PMCV), piscine orthoreovirus (PRV) and salmon gill poxvirus (SGPV).

Parent fish were pre-screened for the equivalent, apart from SGPV.

2.3. SAV3 inoculum

The SAV3 originated from pooled heart and head kidney samples of Atlantic salmon from the Hordaland region of Norway (Taksdal et al. 2015). Propagation of SAV3 was performed using the CHSE-214 cell line (ATCC® CRL-1681™), which was derived from a Chinook salmon *Oncorhynchus tshawytscha* embryo. These cells were grown on L-15 (Leibowitz) medium (Lonza), supplemented with 10% fetal bovine serum (FBS) and gentamicin at 20°C. Serial 10 fold dilutions of the SAV3 stock (passage number 4) were inoculated onto 24 h old CHSE-24 monolayers in 96 well plates, allowing quantification. The viral endpoint titre, measured as 50% tissue culture infective dose ($TCID_{50}$), was determined to be 10^6 $TCID_{50}$ ml^{-1} as described by Reed & Muench (1938).

2.4. Experimental tanks

Three identical 500 l seawater tanks were used in the challenge trial, designated T_L, T_H and T_C for low viral dose, high viral dose and negative control tank, respectively. The seawater originated from 105 m depth from Byfjorden and had been filtered through 20 µm drum filters and treated with UV light (135 W m^{-2}). The water flow in all tanks was the same throughout the experiment, with an average flow rate of 950 l h^{-1} $tank^{-1}$ and was set according to the biomass, dissolved oxygen level and tank water temperature in order to meet an optimal oxygen level for the fish. Water was monitored daily for temperature, salinity and dissolved oxygen levels throughout the challenge. During the challenge period in T_L, T_H and T_C, respectively, the following variations were seen; dissolved oxygen ranged between 79–97, 80–97 and 79–86%, tank water temperatures between 11.7–12.3, 11.7–12.3 and 11.5–12.4°C, and salinity levels between 34.1–34.5, 34.1–34.5 and 34.2–34.5‰.

All tanks had a daily photoperiod of 12 h light:12 h dark, provided by an automatic artificial lighting system. During the 12 h of light, an automatic feeder dispenser fed the fish with 3 mm Nutra Olympic pellets (Skretting). The amount of food given in T_C was between 80–150 g; in T_L, between 56–140 g; and in T_H, between 56–140 g; amounts were adjusted marginally as the fish were growing, dying or being sampled.

Clinical signs in the fish, as well as mortality, were monitored daily in all 3 tanks. Dead experimental fish were removed daily and did not undergo any further analysis.

2.5. Challenge

A total of 45 fish (shedder fish) were immersed into a bath with the anaesthetic Finquel® vet. 1000 mg g^{-1} (100 mg l^{-1}). Once immobilized, each shedder fish was administered with 0.2 ml inoculum by intraperitoneal (i.p.) injection of either a low SAV3 dose of 2×10^2 $TCID_{50}$ $fish^{-1}$, a high SAV3 dose of 2×10^4 $TCID_{50}$ $fish^{-1}$ or virus-free Leibovitz-15 (L-15) cell culture medium containing 2% FBS (mock inoculum). All shedder fish were marked by adipose fin clipping, which allowed us to distinguish them from cohabitant fish. Each group of 15 shedder fish was then transferred into the respective 500 l seawater tank (T_L, T_H and T_C) containing 55 cohabitant fish which had been transferred into the tanks 2 d earlier (–2 d post-challenge, DPC). The shedder fish remained in the tanks throughout the entire challenge period. The challenge was performed and approved in accordance with the Norwegian Animal Research Authority (NARA).

2.6. Sampling

Sampling of tank water and cohabitant fish was performed at 16 different time points: 0, 7, 12, 16, 19, 20, 21, 22, 23, 24, 25, 28, 29, 30, 33 and 37 DPC. Water sampling was carried out by using sterilized 1000 ml Borosilicate 3.3 glass bottles (VWR®) that were submerged by hand to approximately 15 cm below the water surface with the mouth of the bottle turned towards the water current. Five 1 l water samples were taken from each of the 3 tanks at each sampling time point. T_C was sampled first, followed by T_L and lastly T_H.

Three fish were randomly collected from each of T_L and T_H, and one fish was collected from T_C at each sampling point. Sampled fish were euthanized through an immersion bath with an overdose of Finquel® vet. 1000 mg g^{-1} (150 mg l^{-1}). Gross pathology was evaluated, and tissue samples were collected from heart (including the valves and bulbus arteriosus) and mid-kidney for RT-qPCR (stored in RNA*later*™ Soln. [Thermo Fisher Scientific Baltics], at room temperature) and heart and pancreas (pyloric caeca with attached pancreatic tissue) for histopathology (stored in 10% neutral buffered formalin, at room temperature).

2.7. Histopathology

Histopathological examination was performed with a light microscope on pancreas and heart tissue samples from cohabitant fish, identified as SAV3-positive by RT-qPCR, in order to confirm SAV infection and PD. The tissue samples were fixed in 10% neutral buffered formalin and processed according to standard procedures at the Norwegian Veterinary Institute in Oslo (NVI, Oslo).

2.8. Concentration of SAV3 from tank water samples

Concentration of SAV3 from 1 l water samples from each of the 3 water tanks was performed according to 5 CMs, previously developed by Weli et al. (2021). Briefly, the methods involved filtration of 1 l tank water through either an electropositive or an electronegative membrane filter, followed by elution of the adsorbed material from the filter with one of 4 different buffer solutions (buffer 1–4). An overview of the 5 filter/buffer combinations is given in Table 1. Filters were then inserted into a 47 mm in-line filter holder (Merck Millipore) fitted to a Masterflex® portable environmental sampler pump (Cole-Parmer Instrument Company). Following filtration of the water sample, the filter was rinsed using a buffer solution.

For the methods using buffer 1, the intact filter was rinsed in a 50 mm Petri dish with 2.4 ml buffer 1 and subsequently shaken on an orbital shaker at 600 rpm for 30 min. For the 3 other buffer solutions, the filter was cut into <1 cm^2 fragments and transferred into a 50 ml CELLSTAR® tube (Greiner Bio-One) containing 4.0 ml buffer 2, 3 or 4 and subsequently vortexed 3 times for 1 min with 5 min rests. The eluate (i.e. tank water concentrate) was distributed as 1 ml into 1.5 ml Eppendorf® SafeSeal tubes (Sarstedt AG & Co.) and stored at −80°C until RNA extraction.

Tank water from T_C was filtered first, followed by T_L and lastly T_H. Hoses and filter heads were disinfected between sampling of each tank by pumping (flow rate 0.2 l min^{-1}) 1 l of 10% chlorine for 10 min, followed by neutralisation with 1 l of 10% Alfa Aesar sodium thiosulfate pentahydrate, 99+% (Thermo-Fisher Scientific) for 10 min and finally by rinsing with 1 l distilled water for 10 min.

2.9. RNA extraction of tank water concentrates

One ml NucliSENS® easyMAG® Lysis Buffer (bioMérieux) was added to 1 ml tank water concentrate followed by RNA extraction, using the easyMAG® robot (bioMérieux) and the standard lysis protocol (generic 2.0.1.), which was performed with 50 µl magnetic silica beads, according to the NucliSENS easyMAG user manual (bioMérieux 2009). RNA was eluted in 50 µl buffer and stored at −80°C until use in RT-qPCR.

2.10. RNA extraction from organ tissue

Tissue samples (i.e. heart and mid-kidney) were stored in RNA*later*™ Soln. (Thermo Fisher Scientific Baltics) at −80°C prior to RNA extraction, which was performed by adding approximately 20 mg tissue with 180 µl ATL Lysis Buffer (Qiagen®) and 20 µl Proteinase K and incubation overnight at 56°C. Extraction was performed with QIAcube® (Qiagen®) with the reagents from the DNeasy Blood & Tissue Kit (Qiagen®), giving an RNA elution volume of 200 µl. Isolated RNA was stored at −80°C until use in RT-qPCR.

Table 1. Overview of the 5 different concentration methods ($CM_{A–E}$) used to concentrate salmonid alphavirus subtype 3 (SAV3) from tank water during the cohabitant challenge trial with post-smolt Atlantic salmon. The methods are presented as 5 different combinations of membrane filters and buffer solutions used for SAV3 adsorption and elution, respectively

Concentration method	Filter	Buffer no.	Buffer solution
CM_A	Electronegative[a]	1	Lysis buffer[c]
CM_B	Electropositive[b]	1	Lysis buffer[c]
CM_C	Electronegative[a]	2	1 mM NaOH[d] (pH 9.5)
CM_D	Electronegative[a]	3	L-15 medium[e] (pH 9.0) + 2% FBS
CM_E	Electropositive[b]	4	L-15 medium[e] (pH 7.3–7.75) + 2% FBS

[a]Electronegative MF-Millipore™ 0.45 µm MCE membrane (Merck Millipore); [b]Electropositive Zeta Plus™ 1 MDS membrane (3M Purification); [c]NucliSENS® easyMAG® Lysis Buffer (bioMérieux); [d]1 mM NaOH (Sigma-Aldrich); [e]L-15 (Leibovitz) Medium (Lonza)

2.11. RT-qPCR

The SAV3 strain was detected using the Q_nsP1 assay (Hodneland & Endresen 2006). This broad-spectrum assay detects all known SAV subtypes using primers and probe with final concentrations of 500 and 300 nM, respectively and amplifies a conserved region in the 5' end of the Q_nsP1-gene, giving amplicons of 107 bp (Table 2).

Extracted RNA was automatically pipetted by Eppendorf epMotion® 5075 (Eppendorf) in duplicates, analyzed by RT-qPCR on an AriaMx machine (Agilent Technologies) and evaluated with the Agilent AriaMx Real-Time PCR software (version 1.7). Each plate included a negative control sample and an inter plate calibrator of pure SAV3 RNA, which were both run in duplicates.

The cut-off quantification cycle (Cq) value was set to 40; samples with values below this Cq in duplicates were considered positive. Samples with only one positive parallel were rerun and considered positive only with positive duplicates. The template volume was 2.0 µl RNA in a total reaction volume of 20 µl, and the RT-qPCR kit used was TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems®). The thermal programme comprised reverse transcription for 5 min at 50°C and enzyme activation for 2 min at 95°C, followed by 45 cycles of 15 s at 94°C and 40 s at 60°C. GraphPad Prism 4.03 (GraphPad Software) was used to plot the data.

2.12. Check of RNA purity

All tank water concentrates and tissue samples were evaluated for inhibitors that could impact the RT-qPCR and target quantification. RNA was, therefore, analyzed undiluted (1:1) and diluted (1:4) in duplicates, by RT-qPCR. Potential inhibition was detected when the Cq difference between the 1:1 and 1:4 samples was found to be less than 2 Cq. For these samples, the 1:4 dilution was used to estimate virus quantities.

2.13. Quantification of SAV3

A sample of purified SAV3 RNA (inter plate calibrator) was quantified by RT-ddPCR. The RT-ddPCR analysis was performed as described by Weli et al. (2021), by using the One-step RT-ddPCR Advanced Kit for Probe (Bio-Rad Laboratories), and the primers and probe used for the RT-qPCR assay, with the final concentrations of 900 and 250 nM, respectively.

A 2-fold serial dilution (1:1 to $1:2^{12}$) was run by RT-qPCR as a standard curve. The amplification efficiency (E = 94%), correlation coefficient (r^2 = 0.995) and slope of the linear regression line were all evaluated in accordance with Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Taylor et al. 2010).

Quantification of SAV3 particles was performed based on the following formula: $N_1 = N_2 \times (1 + E)^{(Cq_2 - Cq_1)}$ (Christensen et al. 2017), where N_1 and N_2 represent the SAV3 copy number in the unknown sample and the calibrator, respectively and Cq_1 and Cq_2 represent the SAV3 detection in Cq values in the unknown sample and the calibrator, respectively. In order to estimate the number of viral particles in 1 l of tank water concentrated with the first CM (CM_A), copy numbers were multiplied with $25 \times 2.4 \times 1/R$ where R (recovery) is approximately 25%, as calculated according to Weli et al. (2021). The data were $\log_{10}$ transformed and plotted in GraphPad Prism 4.03 (GraphPad Software).

2.14. Limit of detection and limit of quantification of SAV3

The limit of quantification (LOQ) and limit of detection (LOD) values for the RT-qPCR were calculated using the following formulas: $LOQ = 10\sigma/S$ and $LOD = 3.3\sigma/S$, where σ is the standard deviation of y-intercepts from 2 SAV3 standard curves and S is the slope of the curve, according to the ICH Q2 (R1) guidelines (EMEA 1995). LOD and LOQ for SAV3 in 1 l of tank water, using the CM_A method, was estimated as described in Section 2.13.

Table 2. The Q_nsP1 assay was used for the detection of salmonid alphavirus subtype 3 (SAV3) in tissue samples and tank water concentrates, from the cohabitant challenge trial with post-smolt Atlantic salmon

Oligonucleotide	Sequence	Position	Amplicon length (bp)	Reference strain	Reference
Q_nsP1 F primer	5'-CCG GCC CTG AAC CAG TT-3'	17–33	107	AY604235	Hodneland & Endresen (2006)
R primer	5'-GTA GCC AAG TGG GAG AAA GCT-3'	54–69			
Probe	FAM-5'-CTG GCC ACC ACT TCG A-3'-MGB	103–123			

2.15. Statistical comparisons of Cq values

In order to determine whether there was a statistically significant difference in performance between the 5 CMs, pairwise comparisons of the CMs were performed in one analysis (Model 1), and between the 2 membrane filters and the 4 buffer solutions with another (Model 2). Post hoc Tukey's HSD tests were used to achieve this goal. The performance was measured as Cq values from the RT-qPCR. Cq values were log transformed prior to the regressions to approximate normality. Cq values above 40 (i.e. no detection of SAV3 RNA) may constitute a problem for the assumption of normality, and were therefore removed. The virus was only detected in 2 observations in T_L using CM_E and was therefore clearly not suited to extract SAV3 RNA in this case, hence it was removed from the data set. Non-linear temporal autocorrelation was handled by modelling sampling time points (i.e. DPC) as a third-degree polynomial, since common approaches like a first-order autoregression (AR1) and autoregressive moving-average (ARMA) are not warranted for sampling designs with uneven sampling intervals. The inclusion of DPC will handle the development of the disease over time, as well as the removal of fish for tissue analysis. A forward model selection procedure was used to evaluate the inclusion of explanatory variables, using Akaike's information criterion (AIC) as an optimization criterion, and interactions were evaluated after the fixed effects were included. The best and the next best models were compared using an ANOVA table function in R. Explanatory variables, tried in the model selection for Model 1, were DPC (as a third-degree polynomial), viral dose and CM. Explanatory variables tried in model selection for Model 2 were DPC (as a third-degree polynomial), viral dose, filter and buffer. The model was validated using a cross validation procedure: the data set was randomly split into training data (80% of the data set) and validation data (20%). The model was refitted on the training data and used to predict the Cq values in the validation data set; predicted and observed validation data was stored. This procedure was repeated 10 000 times. Predictive R^2 values were calculated as the squared Pearson correlation coefficient between observed and predicted Cq values, and were used as a measure of how well the model is able to predict observations removed from the data set, in order to evaluate model overfitting in the linear models.

Associations between log-transformed Cq values in tank water concentrates and tissue (heart or midkidney) in both viral dose tanks (T_L and T_H) were estimated using a mixed effect linear model approach (Model 3) and backwards model selection, using AIC as an optimization criterion. The best and the second best models were compared using an ANOVA table function in R. Temporal autocorrelation and repeated measures (3 fish analyzed from each tank at each sampling time point) were handled by including DPC as a random intercept term, and viral dose tanks (T_L and T_H) as a fixed effect term. As a single water sample was analyzed by each CM at each sampling time point, sampling repeatability was deducted from the variability in the statistical model. This was done by simulating 1000 data sets from the model (using the 'simulate()-' function in R), and investigating the distribution of the simulated samplings.

All analyses were performed using the R statistical software (version 3.6.2), and multiple comparisons (Tukey's HSD test) based on the linear model were fitted using the 'multcomp' package (Hothorn et al. 2008). Mixed effect models were fitted using the 'lme4' package (Bates et al. 2015).

3. RESULTS

3.1. Clinical signs

Clinical signs associated with PD included an overall reduced appetite (i.e. failure to thrive), lethargy and abnormal swimming behavior and findings of faecal casts and mucus in the tank water. These signs were observed for the first time at 12 DPC in the high viral dose tank (T_H) and at 16 DPC in T_L (Fig. 1).

3.2. Mortality

One dead cohabitant fish was observed in T_L, at 20 DPC, whereas no cohabitant fish died in T_H until the last day of the challenge (1 fish died at 37 DPC; Fig. 1). In T_C, no fish died during the challenge period.

3.3. Gross pathology

Characteristic gross pathological changes associated with PD in cohabitant fish, including yellow mucoid gut content, empty intestines, ascites and petechial haemorrhages in the fat surrounding the pyloric caeca, were observed for the first time at 16 DPC in T_H and at 20 DPC in T_L, and thereafter

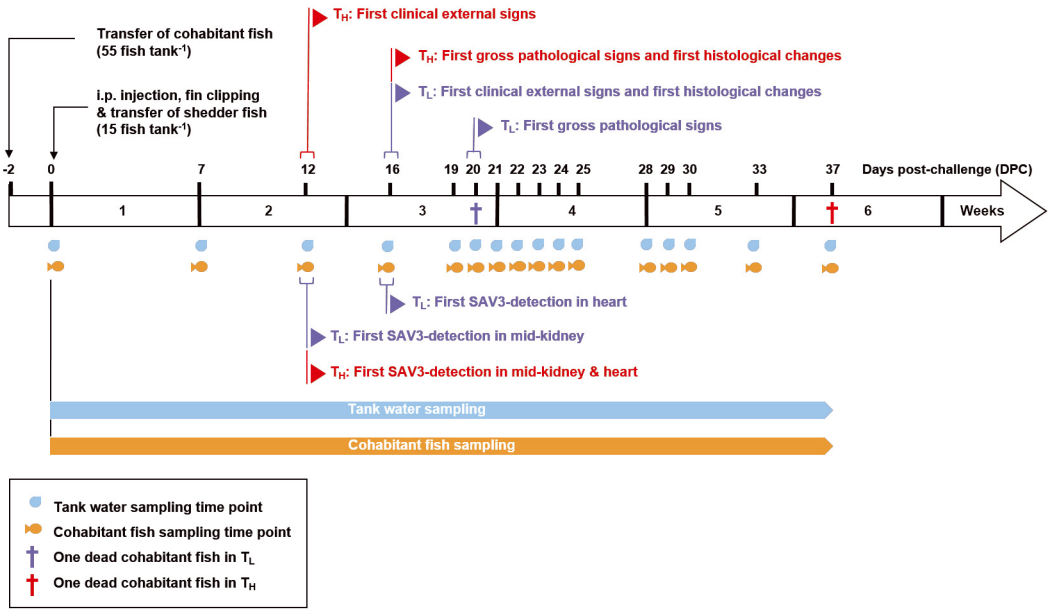

Fig. 1. Timeline for the salmonid alphavirus subtype 3 (SAV3) cohabitant challenge trial with post-smolt Atlantic salmon showing the 16 sampling time points, given as days post-challenge, for tank water and cohabitant fish. At Day 0, shedder fish were administered with a 0.2 ml SAV3 inoculum by intraperitoneal (i.p.) injection and subsequently fin clipped prior to their distribution between the 3 different tanks: the low viral dose tank (T_L: 2×10^2 50 % tissue culture infective dose [$TCID_{50}$] $fish^{-1}$), the high viral dose tank (T_H: 2×10^4 $TCID_{50}$ $fish^{-1}$) and the negative control tank (T_C: virus-free L-15 [Leibovitz] medium)

observed at every sampling time point in almost all sampled fish in both T_L and T_H. Additionally, cohabitant fish collected from T_L had a more severe gross pathology associated with PD in the beginning of the challenge period compared to fish collected from T_H. For comparison, cohabitant fish from T_C were also evaluated and had normal autopsy findings throughout the challenge.

3.4. Histopathology

Tissue samples from the cohabitant fish collected from T_L and T_H started presenting typical histopathological signs associated with SAV infection at 16 DPC, including necrosis and severe loss of exocrine pancreatic tissue and focal myocardial degeneration, evaluated by haematoxylin and eosin (H&E) staining (except for the cardiac tissue sections from 3 cohabitants collected from T_L at 16 DPC). Different levels of histopathological changes typical of SAV infection were found at all later sampling time points. For comparison, cohabitant fish collected from T_C were evaluated at all sampling time points and presented intact and normal pancreatic and cardiac muscle tissues. The pancreatic tissue sections from the cohabitant fish sampled from T_L and T_H between 16, 29 and at 37 DPC (one cohabitant fish from T_L) were positive by immunohistochemistry (IHC) staining for SAV, indicating acute SAV infection on these days.

3.5. Detection of SAV3 in tissue samples

A total of 94 tissue samples (2 were lost) from cohabitant fish from each of T_L and T_H, and 32 from T_C were analyzed by RT-qPCR. A majority were found to contain inhibition. Inter-individual variability was observed in the concentration of SAV3 RNA copy numbers detected in the tissue samples (mid-kidney and heart), in both T_L and T_H. SAV3 was first detected in T_H at 12 DPC for both mid-kidney (Cq 27.9) and heart (Cq 27.0) (Fig. 2B), and in T_L at 16 DPC for both mid-kidney (Cq 19.0) and heart (Cq 21.1) (Fig. 2A). Peak levels of SAV3 detection in the mid-kidney were seen at 19 DPC in T_L (Cq 14.7) and at 16 DPC in T_H (Cq 19.9) (Fig. 2A,B). Peak levels of SAV3 detection in the heart were seen at 23 DPC in T_L (Cq 15.0) and at 21 DPC in T_H (Cq 14.9) (Fig. 2A,B). On the last sampling time point (37 DPC), the highest SAV3 concentration was detected in the heart in T_L (Cq 17.7), followed by the mid-kidney in T_L (Cq 23.0), the heart in T_H (Cq 23.1) and the mid-kidney in T_H (Cq 28.3). SAV3 was detected in all tissue samples from the onset until (and including) the last day of challenge (37 DPC) in both T_L and T_H.

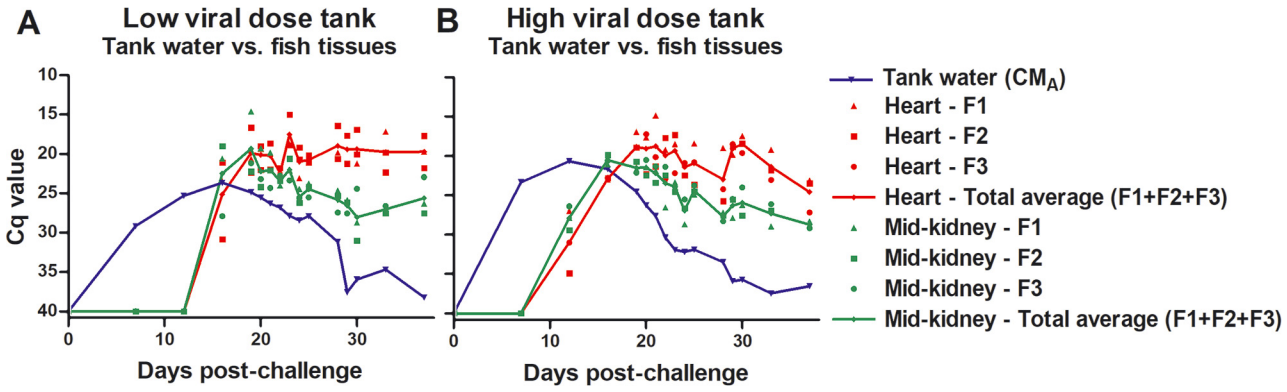

Fig. 2. Comparison between detection of salmonid alphavirus subtype 3 (SAV3) in 1 l tank water concentrated with method A (CM_A) and in tissue samples (mid-kidney and heart) from cohabitant fish sampled during the SAV3 cohabitant challenge. The samples were analysed by RT-qPCR. The SAV3 concentration is presented as the average quantification cycle (Cq) values per days post-challenge in the (A) low viral dose tank (T_L) and (B) high viral dose tank (T_H). Tank water samples were collected simultaneously with the collection of the 3 cohabitant fish $tank^{-1}$ (F1: fish 1; F2: fish 2; F3: fish 3) at 16 sampling time points, from both T_L and T_H

Tissue samples collected from T_C were found to be negative by RT-qPCR.

3.6. Detection of SAV3 in tank water samples

Eighty 1 l water samples were collected from each of the 3 tanks (T_L, T_H and T_C), resulting in a total of 240 samples that were analysed by RT-qPCR, and the majority of these samples were found to contain inhibitors. The first detection of SAV3 was made on the second sampling time point (7 DPC) in both T_L and T_H, with all CMs except CM_E.

3.7. Comparison of the 5 CMs ($CM_{A–E}$)

Compared with the other 4 CMs, Fig. 3 revealed a general tendency for CM_A, the combination of electronegative filter with buffer 1, to be best in recovering SAV3 from 7 DPC until the end of challenge. This tendency can be observed in both T_L and T_H, but with a stronger tendency in the latter (Fig. 3A,B). Additionally, the period of virus detection was narrower and had a higher detection peak in the T_H compared to T_L (Fig. 3C,D). When using CM_A, the first SAV3 detection in tank water was made at 7 DPC in both T_L and T_H; the peak of viral shedding occurred earlier in T_H (12 DPC), with 3.42×10^6 SAV3 RNA copies l^{-1} (6.53 $\log_{10}$ copies l^{-1}), than in T_L (16 DPC), with 4.7×10^5 SAV3 RNA copies l^{-1} (5.67 $\log_{10}$ copies l^{-1}) (Fig. 3C,D). Water samples collected from T_C were negative, except for one sample collected at 19 DPC (Cq 34.6), when using CM_D. The simulated samplings from the statistical model showed that the Cq values varied between 19.7 and 45.0 for CM_A in both tanks over the entire course of the challenge. More than 99% of these simulated samplings returned Cq values below 40, indicating that we would have detected SAV in more than 99% of the samples at any time point after 7 DPC.

The best fitted model for CM (Model 1) included a CM, DPC as a third-degree polynomial (allowing non-linear time trends in the development of the infection during the challenge period) and viral dose (ANOVA model comparison: $p < 0.001$). The model also included interactions between CM and viral dose, and between viral dose and DPC, allowing a slower development of the infection and a lower peak in T_L. The model became quite complex, and overfitting was a serious concern. The predicted R^2 from cross validation (0.78) was close to the multiple R^2 (0.84), proving that the model can predict observations that were excluded from the training data with good precision. Pairwise comparisons revealed that CM_A returned significantly lower Cq values than all the other CMs when SAV3 was detected in T_H (Table 3; CM_A: p-values between 0.004 and <0.001), but CM_A was not different from CM_B in T_L ($p = 0.988$).

The best fitted model for filter and buffer (Model 2) included filter, buffer, DPC (as a third-degree polynomial) and viral dose, in addition to interactions between viral dose and DPC, and between viral dose and filter (ANOVA model comparison: $p < 0.001$). The predicted R^2 (0.78) was close to the multiple R^2 (0.84), proving that the model can predict observations that were excluded from the training data with

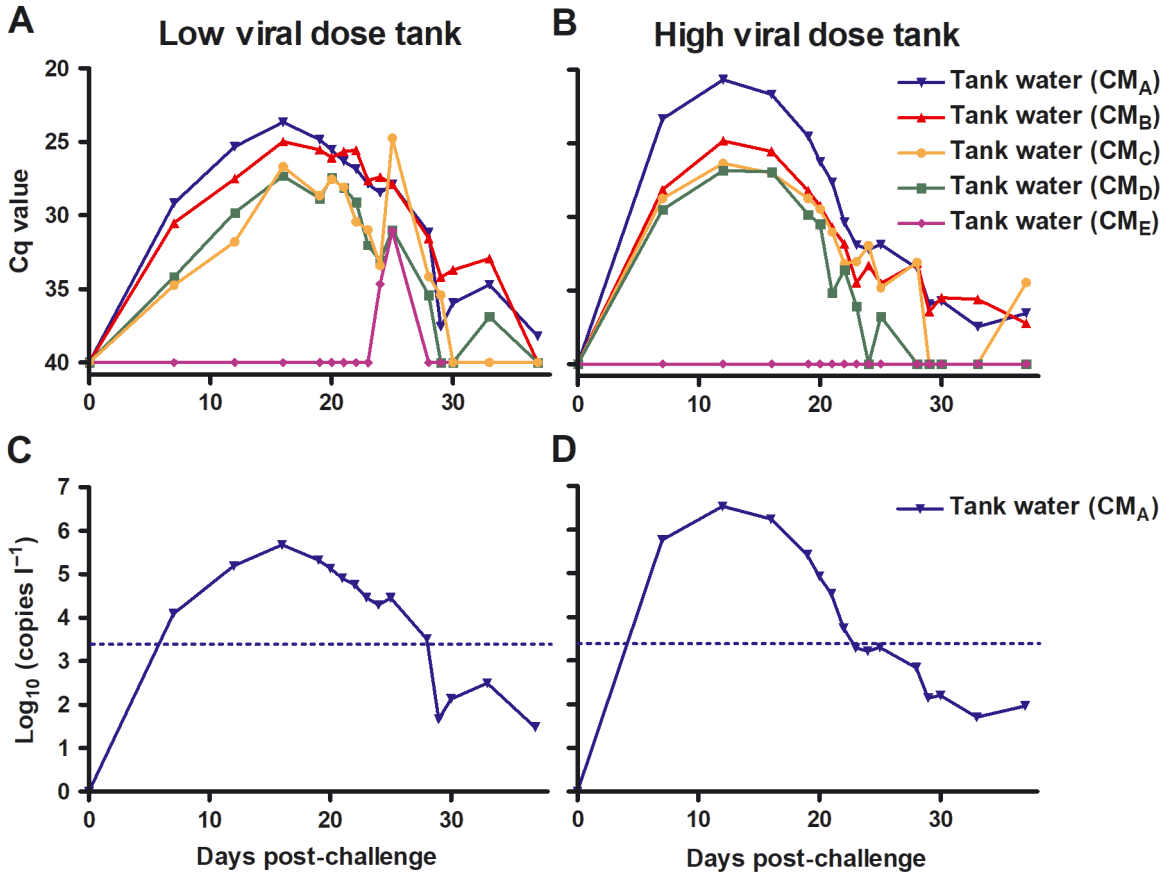

Fig. 3. Detection of salmonid alphavirus subtype 3 (SAV3) from tank water sampled at 16 time points, given as days post-challenge (DPC) throughout the SAV3 cohabitant challenge trial with post-smolt Atlantic salmon. The samples were analyzed by RT-qPCR. Five methods ($CM_{A–E}$) were used to concentrate SAV3 from 1 l of tank water in the low viral dose tank (T_L) and high viral dose tank (T_H). Both (A,B) quantification cycle (Cq) values per DPC and (C,D) SAV3 copies l^{-1} of tank water DPC^{-1} are presented. Dotted horizontal line: limit of quantification when using CM_A

good precision. Pairwise comparisons revealed that buffer 1 performed significantly better than buffers 2 and 3 ($p < 0.001$ for both comparisons), and the electronegative filter performed significantly better than electropositive for T_H ($p = 0.002$), but not for T_L ($p = 0.91$) (Table 3).

3.8. LOD and LOQ of SAV3 in 1 l of tank water

The LOD and LOQ when using CMA were 902 and 2736 SAV3 RNA copies l^{-1}, respectively.

3.9. Detection of SAV3 in tank water concentrates vs. tissue samples

Associations between virus recovery in tissue samples and tank water concentrates were explored using a linear mixed effect model (Model 3). The model was fitted using a backwards model selection (AIC). The full model (before model selection) explained the tank water concentrates' Cq values for SAV3 detection as the effect of DPC (as a random intercept term), viral dose (as a factor variable), tissue samples' Cq values for SAV3 detection and the interaction between the Cq values for tissue samples and dose. Any removal of explanatory terms gave a poorer fit, and the full model was therefore kept (confirmed by the ANOVA model comparison, $p = 0.02$). The model output is presented in Table 4. A significant positive association was established between the Cq values for mid-kidney samples and tank water concentrates ($p = 0.0084$) in both T_L and T_H. The effect of viral dose and the interaction were significant ($p < 0.038$ and $p = 0.024$, respectively). No significant effect was found with a similar approach for heart tissue samples ($p = 0.26$).

Table 3. Post hoc Tukey HSD tests, based on the linear models (Models 1 and 2), exploring how the quantification cycle (Cq) values for the detection of salmonid alphavirus subtype 3 (SAV3) could be explained by the concentration method (CM), the 2 membrane filters (electropositive or electronegative) and the 3 buffer solutions (buffers 1, 2 and 3) used for the concentration of SAV3 from 1 l tank water. The columns indicate the null hypotheses, the mean differences and the p-values. Note that there was an interaction between CM and viral dose in both models. Tukey comparisons (contrasts) in these cases are shown for detected SAV3

Tukey null hypotheses	Mean difference of log(Cq)	p
CM in high viral dose tank (model 1)[a]		
$CM_B - CM_A = 0$	0.073	0.004
$CM_D - CM_A = 0$	0.16	<0.001
$CM_C - CM_A = 0$	0.092	<0.001
$CM_D - CM_B = 0$	0.088	0.003
$CM_C - CM_B = 0$	0.019	0.820
$CM_C - CM_D = 0$	−0.069	0.040
CM in low viral dose tank (model 1)[a]		
$CM_B - CM_A = 0$	−0.0069	0.988
$CM_D - CM_A = 0$	0.098	<0.001
$CM_C - CM_A = 0$	0.087	0.001
$CM_D - CM_B = 0$	0.10	<0.001
$CM_C - CM_B = 0$	0.094	<0.001
$CM_C - CM_D = 0$	−0.011	0.968
Filter and buffer (model 2)[a]		
Electropositive – Electronegative, high viral dose	−0.064	0.002
Electropositive – Electronegative, low viral dose	0.0023	<0.909
Buffer 1 – Buffer 3 = 0	−0.13	<0.001
Buffer 2 – Buffer 3 = 0	−0.036	0.101
Buffer 2 – Buffer 1 = 0	0.089	<0.001

[a]See Table 1

4. DISCUSSION

In the present study, we present a successful CM for detection of SAV3 in seawater. We have previously demonstrated the potential of this method for recovery of SAV3 spiked into natural seawater (Weli et al. 2021). However, in order to optimise the method for the field, 5 CMs ($CM_{A–E}$), based on 2 different membrane filters and 4 different buffer solutions, were evaluated for their ability to recover SAV3 from tank water collected during a cohabitant challenge trial, when post-smolt Atlantic salmon had developed clinical infection with SAV.

The method combining the electronegative membrane filter and NucliSENS® easyMAG® Lysis Buffer (CM_A) had the highest SAV3 recovery and the most consistent SAV3 detection in tank water samples from T_H throughout the challenge, but virus recovery using CM_A or CM_B was indistinguishable in samples from T_L. This buffer solution and the electronegative membrane filter performed significantly better compared to the other buffer solutions and the electropositive filter used herein, although the filters were indistinguishable in samples from T_L. This conclusion is also consistent with the above-mentioned *in vitro* study, in which we concluded that the same combination of the electronegative filter and Nucli SENS® easyMAG® Lysis Buffer provided the best SAV3 recovery from seawater (Weli et al. 2021). The method combining the electropositive filter and NucliSENS® easyMAG® Lysis Buffer (CM_B) also resulted in higher SAV3 recovery compared to the other 3 CMs (CMc, CM_D and CM_E), thus strengthening the importance of the NucliSENS® easyMAG® Lysis Buffer, as stated by our previous *in vitro* study (Weli et al. 2021).

Horizontal transmission of SAV via water has been confirmed by other experimental challenge studies, in which SAV was detected directly from seawater using filtration (Andersen et al. 2010, Jarungsriapisit et al. 2016a,b, 2020). In these studies, an electropositive 1 MDS filter was used for filtration of 1 l seawater, followed by rinsing of the filter using lysis buffer (Andersen et al. 2010, Jarungsriapisit et al. 2016b) or L-15 supplemented with 10% FBS (Jarungsriapisit et al. 2016a, 2020). In other words, none of these studies used the filter/buffer combination (CM_A) that proved most successful in the present study. However, in this context it is important to highlight that the subsequent processing of the filtrate (i.e. RNA extraction) and the PCR assay used can also impact on the method's success in detecting SAV3 from seawater.

Although the cohabitation challenge model, as used in this study, does not represent the natural route of infection (since the initiation of infection was done via i.p. injection of shedder fish), it does simulate spread of the virus and a natural exposure route to the cohabitant fish.

McLoughlin & Graham (2007) suggested that the incubation period for SAV may be 7–10 d at seawater temperatures of 12–15°C. In the present study, the tank water temperature was approximately 12°C, and SAV3 was first detected in the tank water at 7 DPC, compared to in cohabitant fish tissue at 16 DPC in T_L and at 12 DPC in T_H. However, the early detections of SAV3 in the tank water may have been

Table 4. The most parsimonious model explaining the association between quantification cycle (Cq) values for detection of salmonid alphavirus subtype 3 (SAV3) in the mid-kidney samples and in the tank water concentrates. Only fixed effects from the model are shown. Note that Cq values are log transformed in the response; '×' means interaction. Baseline for the model output is high dose. This means that the intercept and log(mid-kidney × Cq-values) from the table are parameters in a linear function between log(tank water Cq values) and log(mid-kidney Cq values) in samples from the high viral dose tank. In this case the model takes the form '$a + bx$', where 'a' is the intercept estimate and 'b' (the slope) is the log(mid-kidney Cq values) estimate. The estimate for low viral dose is presented as the deviation from the intercept for high viral dose, and the 'log(mid-kidney Cq values) × low viral dose' estimate is presented as the deviation from the slope for high viral dose. The 'a' in the linear function for low viral dose becomes the intercept + low viral dose estimates, and 'b' is the log(mid-kidney Cq values) + log(mid-kidney Cq values) × low viral dose estimates

Variable	Estimate	SE	p
Intercept	2.7	0.27	< 0.001
log(mid-kidney Cq values)	0.22	0.081	< 0.008
Low viral dose	−0.51	0.24	< 0.038
log(mid-kidney Cq values) × low viral dose	−0.17	0.076	0.024

virus excreted from the shedder fish that remained in the tanks throughout the challenge.

In contrast to the *in vitro* study (Weli et al. 2021), the present study involved filtration of tank water that contained a high amount of organic matter (e.g. faecal casts, mucus and pellets). Furthermore, the tank water samples were collected approximately 15 cm below the water surface of the tanks, and a previous study (Stene et al. 2016) has identified accumulated levels of SAV in surface lipids due to fat droplets leaking from dead and SAV-infected fish. Hence, the detected SAV3 from the tank water in the present study could have been influenced by the inevitable inclusion of the fat droplets and/or organic matter (e.g. faecal casts and mucus) that have been found positive for SAV3 RNA, as shown by previous research (Graham et al. 2011).

In the present study, SAV3-infection in cohabitant fish was confirmed by gross pathology, histopathology and clinical signs associated with PD (Nelson et al. 1995, McLoughlin et al. 1996, 2002, Taksdal et al. 2015, Jansen et al. 2017), as well as by RT-qPCR analysis.

Heart and mid-kidney, which are the recommended organs for SAV detection according to the World Organisation for Animal Health (OIE) and are used in the Norwegian surveillance program (Lovdata 2017), were sampled from cohabitant fish in the present study. A significant positive correlation was found throughout the challenge between the levels of SAV3 in the tank water samples and in the mid-kidney samples collected from the cohabitant fish in both T_L and T_H. This observation supports that the cohabitant fish provided a significant contribution to virus levels in the tank water throughout the challenge. No correlation was found between virus concentrations in the tank water samples and the heart samples, either in T_L or in T_H. However, the virus concentrations were generally found to be higher in the heart samples compared to the mid-kidney.

Tank water samples were collected consistently from all 3 tanks before the collection of the cohabitant fish, ensuring that the fish would not be exposed to stress that might cause them to increase their shedding rate prior to the water sampling. The water flow rate in all 3 tanks was constantly high (950 l h^{-1}), ensuring not only optimal oxygen levels for the fish, but also providing self-cleaning of the tanks throughout the challenge. Hence, it is highly suspected that the one water control which was found to be SAV3-positive when using CM_D was due to cross-contamination during sampling or sample processing.

Monitoring for the presence of SAV3 in the tank water sampled during the challenge period was done by RT-qPCR, which enables high performance in detection of waterborne RNA viruses at low concentrations (Girones et al. 2010, Rački et al. 2014a,b). RT-qPCR has been reported to be more sensitive to inhibitors than qPCR (Girones et al. 2010), which may cause lower quantification precision (i.e. larger coefficients of variation) (Rački et al. 2014a). Since the present study involved sampling of natural seawater, in which both salts and other RT-qPCR inhibitors are expected to have an influence on RNA-virus quantification, a check of inhibition was made for all tank water samples, and inhibition was indeed present in a majority of the samples (hence the 1:4 dilution was considered for these). Therefore there is an advantage of using RT-ddPCR on these samples, as this method is less sensitive to any effect of inhibitors (Rački et al. 2014a,b). The method is more expensive compared to RT-qPCR, but the samples could then be run on undiluted RNA only.

The SAV3 concentrations (based on Cq values) in tissue samples were higher overall compared to in-tank water samples. This is an expected finding because the fish (in contrast to the water) serve as a replication site for the virus. However, in this context, it is important to highlight that the detection method

used for tissue is different than the method used for water, and therefore these methods are not comparable in terms of differences in the amount of detected virus.

In laboratory as well as in field studies, the sensitivity of the detection methods should be evaluated according to whether the method is able to detect the virus when there are SAV3-infected fish in the population. In order to compare the sensitivity of the water sampling method to a surveillance method based on sampling of fish under field conditions, the comparison will be between the LOD for the water sampling method on the one side, and the probability of sampling an infected fish on the other. For both methods, the sensitivity will obviously depend on the prevalence of SAV3-infected fish in the population. However, a water sample serves as a representation of the environment of a large number of fish, and if the water sampling method can detect the virus at a low prevalence of SAV in the fish population, it may increase the probability for early detection of SAV in a fish farm.

Based on these findings, the combination of an electronegative charged filter (MF-Millipore™ 0.45 µm MCE membrane; Merck Millipore) and NucliSENS® easyMAG® Lysis Buffer (bioMérieux) is considered to have the best potential in serving as a more cost-efficient, straightforward, rapid, reliable, reproducible and animal-welfare friendly method for concentration and detection of SAV3 and potentially other SAV subtypes from seawater. This new method will be tested for surveillance of farmed salmonid populations as a part of a biosecurity plan for SAV under natural field conditions. The method might allow warning and earlier implementation of disease control measures on farms neighbouring farms with identified SAV, which would be of significance in Atlantic salmon health management.

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