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Development and evaluation of a method for concentration and detection of salmonid alphavirus from seawater

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ABSTRACT

Waterborne viral infections represent a major threat to fish health. For many viruses, understanding the interplay between pathogens, host and environment presents a major hurdle for transmission. *Salmonid alphavirus* (SAV) can infect and cause pancreas disease (PD) in farmed salmonids in seawater. During infection, SAV is excreted from infected fish to the seawater. We evaluated two types of filters and four different eluents, for concentration of SAV3. One L of seawater was spiked with SAV3, followed by filtration and virus elution from membrane filters. For the negatively charged MF hydrophilic membrane filter (MF-) combined with NucliSENS® lysis buffer the SAV3 recovery was 39.5 ± 1.8 % by RT-ddPCR and 25.9 ± 5.7 % by RT-qPCR. The recovery using the positively charged 1 MDS Zeta Plus® Virosorb® membrane filter (MD+), combined with NucliSENS® lysis buffer was 19.0 ± 0.1 % by RT-ddPCR and 13.3 ± 3.8 % by RT-qPCR. The limits of quantification (LOQ) and detection (LOD) were estimated to be 5.18×10^3 and 2.0×10^2 SAV3 copies/L of natural seawater, by RT-ddPCR. SAV3 recovery from small volumes of seawater, and the requirement for standard laboratory equipment, suggest the MF-filter combined with NucliSENS® lysis buffer would be a candidate for further validation in experimental trials.

1. Introduction

Salmonid alphavirus (SAV) is a positive-sense single-stranded RNA virus that belongs to the family *Togaviridae*, genus Alphavirus and encodes structural (E1-E3 and capsid) (Fringuelli et al., 2008), and non-structural proteins nsP1–nsP4 (Weston et al., 1999). The virus is divided into six subtypes (SAV 1–6), (Fringuelli et al., 2008; Hodneland et al., 2005; Karlsen et al., 2006; Weston et al., 1999), and is the causative agent of pancreas disease (PD) in farmed Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Weston et al., 1999). Pancreas disease was first discovered in Scotland in 1976 (Munro et al., 1984), and since then several disease cases have been reported also in other countries (Kent and Elston, 1987; Munro et al., 1984; Murphy et al., 1992; Poppe et al., 1989; Raynard et al., 1992).

In Norway, PD has been diagnosed annually in farmed Atlantic salmon and rainbow trout since the mid-1980s (Olsen and Wangel, 1997). The disease is now considered endemic along the coast of Southwest-, West- and Mid-Norway, while the region of Northern Norway is considered a non-endemic area (Aunsmo et al., 2010; Hjeltnes, 2018). SAV subtype 3 (SAV3) was originally the only SAV subtype known to Norwegian aquaculture. However, since 2011, SAV2 has caused multiple outbreaks of disease in Mid-Norway (Hjeltnes et al., 2016; Hjortaas et al., 2016). The disease occurs mostly during the seawater production phase. Current knowledge indicates that transmission of SAV occurs between neighbouring seawater farms mainly through water currents, while long distance spread is a result of transport of live fish carrying the infection (e.g., smolt, and fish for slaughter) (Haredasht et al., 2019; McLoughlin et al., 2006, 2003; McVicar, 1987; Rodger and Mitchell, 2007; Stene et al., 2014). In Norway, PD has been classified as a notifiable disease since 2007, and control measures have been implemented in order to prevent further spread to areas free of the disease, as well as to reduce losses within the endemic zones. According to revised legislation from 2017, the control of PD requires monthly sampling of fish from all marine farming sites holding salmonid fish for testing by polymerase chain reaction (PCR). This surveillance method leads to sacrificing thousands of fish every year, and is problematic both in relation to aquaculture economy, as well as to animal welfare. For this reason, detection of the virus excreted into the seawater surrounding the fish populations would be more cost effective than sampling of fish, and potentially yield data on virus presence earlier in the course of the

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infection.

Collection of water samples, followed by concentration of dispersed biological material, has been recommended as a method for environmental surveillance, so-called e-DNA (Organization and Initiative, 2015). The method has previously been used on tap water, groundwater, fresh- and seawater to concentrate different pathogens (Abbaszadegan et al., 1993; Logan et al., 1980, 1981; Nupen and Bateman, 1985; Singh and Gerba, 1983; Sobsey et al., 1985). Since salts and other inhibitors in environmental seawater may have influence on virus detection by quantitative RT-PCR (RT-qPCR) (Rački et al., 2014), RT-ddPCR (RT-droplet digital PCR) technology might be favourable. RT-ddPCR has reduced sensitivity to inhibitors and provides direct quantification of the target (Burns et al., 2010; Lui and Tan, 2014; Rački et al., 2014).

In the present study, a first step towards the development of a new surveillance method for SAV in salmonid populations in seawater was initiated. Two different membrane filters and four different elution buffers were evaluated for the concentration of SAV3 from seawater. The virus was spiked into artificial and natural seawater, adsorbed to and eluted from charged filters before quantification with RT-qPCR and RT-ddPCR.

2. Material and methods

2.1. Virus and cell culture

The SAV3 isolate used for this study was originally isolated from pooled heart and head kidney samples of Atlantic salmon from the Hordaland region of Norway (Taksdal et al., 2015). The CHSE-214 cell line (ATCC CRL-1681) derived from a Chinook salmon (*Oncorhynchus tshawytscha*) embryo was used in the propagation of SAV3. Briefly, cells were grown at 20 °C in T-150 culture flasks containing Leibowitz L-15 medium (Life Technologies, UK) supplemented with 10 % foetal calf serum (FBS) and gentamicin (Lonza, USA). The SAV3 isolate was used to inoculate the cell culture, and after seven-days incubation at 15 °C, the culture supernatants were harvested, centrifuged, and 1 mL aliquots were stored at −80 °C before been used for spiking. SAV3 copies in 1 mL of stock (1.62×10^7 copies) used for spiking 1 L of artificial seawater and natural seawater, was quantified by RT-ddPCR.

2.2. Seawater samples

Artificial seawater (Sigma-Aldrich, Germany) in one litre (1 L) polyethylene bottles was stored at 4 °C in the dark before use. Natural seawater in 1 L bottles was obtained from the Oslofjord (kindly provided by the Norwegian Institute for Water Research (NIVA), Solbergstrand research station).

2.3. Filters and buffers

The negatively charged nitrocellulose MF hydrophilic membrane filter (MF-, 47 mm diameter and 0.45-μm pore size) (Millipore, USA) and positively charged 1 MDS Zeta Plus® Virosorb® membrane filter (MD+, 47 mm diameter) (Cuno, Meriden, Conn, USA) were used. In order to develop a new concentration method, preliminary tests were performed to determine the efficiency of MF- and MD + filters for adsorption of SAV3, using the following buffers for elution: (1) NucliSENS® lysis buffer (bioMérieux SA, France); (2) 1 mM NaOH (pH 9.5) buffer; (3) L-15 + 2% FBS (pH 9.0) buffer and (4) L-15 + 2% FBS buffer.

2.4. Initial testing of seawater filtration

The following protocol was used for both MF- and MD + filters. One litre of either artificial seawater or natural seawater were spiked with 1 mL of SAV3 stock (1.62×10^7 copies). The filters were placed into a filter holder, which was fitted to a Masterflex® E/S™ portable environmental sampler (Cole-Parmer Instrument Company, USA), and the water was pumped at a flow rate of 200 mL/min as shown in Fig. 1. After filtration of the spiked seawater, the filter was immediately placed into a Petri dish containing 2.4 mL of buffer 1 and shaken (30 min, 600 RPM, room temperature) to release the membrane-bound virus. For elution with buffer 2, 3 and 4, each filter was cut and placed in a 50 mL Falcon tube containing 4 mL of the respective buffer. Each sample was vortexed (3×1 min, with a 5 min interval at room temperature) and the eluates (concentrates) were stored at −80 °C prior to RNA extraction and SAV3 detection by RT-qPCR and RT-ddPCR. For both types of seawater, a negative control sample without SAV3 spiking was analysed to verify the absence of any natural SAV contaminants. The recovery percentage was calculated based on the formula:

$$\text{SAV3 recovery } (\%) : \frac{SAV3\ particles\ in\ seawater\ concentrate}{SAV3\ particles\ in\ spiked\ seawater} x\ 100$$

As the initial study, using two biological replicates, showed that the MF-/buffer 1 combination gave the highest virus recovery, this combination was further studied in order to assess the efficiency of the method and the limit of detection (LOD) and limit of quantification (LOQ) for SAV3 in 1 L of natural seawater.

2.5. MF-filter concentration method in 1 L of seawater

To further assess the MF-/buffer 1 method for concentration of SAV3 in seawater, a two-fold serial dilution (1:1 to 1: 512) was prepared from the SAV3 stock. Each stock dilution was used to spike 1 L of natural seawater, followed by concentration and elution as described in 2.4. The study was performed four times. RNA was isolated from the spiked seawater concentrate (eluate). SAV3 RNA was quantified using RT-ddPCR and RT-qPCR, and the recovery percentage was calculated.

2.6. Limit of quantification (LOQ) and limit of detection (LOD) of SAV3 in 1 L of natural seawater

In order to estimate the LOQ and LOD for SAV3, a five-fold dilution series (1: 1 to 1: 5^{11}) of the SAV3 stock was prepared. The virus dilutions were used to spike 1 L samples of natural seawater, and the setup was performed twice. The seawater was processed by the MF-/buffer 1 method and RNA extracted from the eluates as described in 2.4. The LOD as estimated by RT-qPCR and RT-ddPCR was given by the lowest amount of SAV3 giving a positive result in the seawater sample, while the RT-ddPCR LOQ was calculated from the highest dilution giving results with a low SD. The SAV3 recovery in the dilution series was also assessed to further evaluate the MF-/buffer 1 method for concentration.

2.7. RNA extraction and RT-qPCR

Total RNA was extracted from 1 mL of SAV3 concentrate from 1 L spiked artificial and natural seawater, according to the generic easyMAG protocol (bioMèrieux, Marcy l'Etoile, France). The RNA was eluted in 50 μL buffer and stored at −80 °C prior to RT-qPCR, using the TaqMan® Fast Virus 1-Step Master Mix kit (Applied Biosystems, USA). The SAV Q-nsP1 primers and probe (F-primer: 5′−CCGGCCCTGAACCAGTT- 3′, R-primer: 5′-GTAGCCAAGTGGGAGAAAGCT-3′ and probe: 5′ FAM-CTGGCCACCACTTCGA-3′ -MGB') were used, which generated a 107-bp PCR-product (Hodneland and Endresen, 2006). A total volume of 20 μL RT-qPCR mix contained 500 nM of each primer, 300 nM probe and 2 μL of RNA. The RT-qPCR reaction was run in duplicates in an Agilent AriaMx PCR System cycler from Applied Biosystems, using the following conditions: reverse transcription at 50 °C for 5 min, initial denaturation at 95 °C for 2 min, and 45 cycles of amplification (94 °C for 15 s, 60 °C for 40 s). Potential inhibition of SAV3 RT-qPCR from the natural seawater was initially evaluated by analysing 1:1 and 1:4 dilutions of the RNA. For samples showing inhibition, the 1:4 dilution was used to estimate virus recovery. Absolute quantification of SAV3 was performed using the formula: $N1{=}N2{*}(1+E)^{(CqN2-CqN1)}$, where N1 and N2 denote

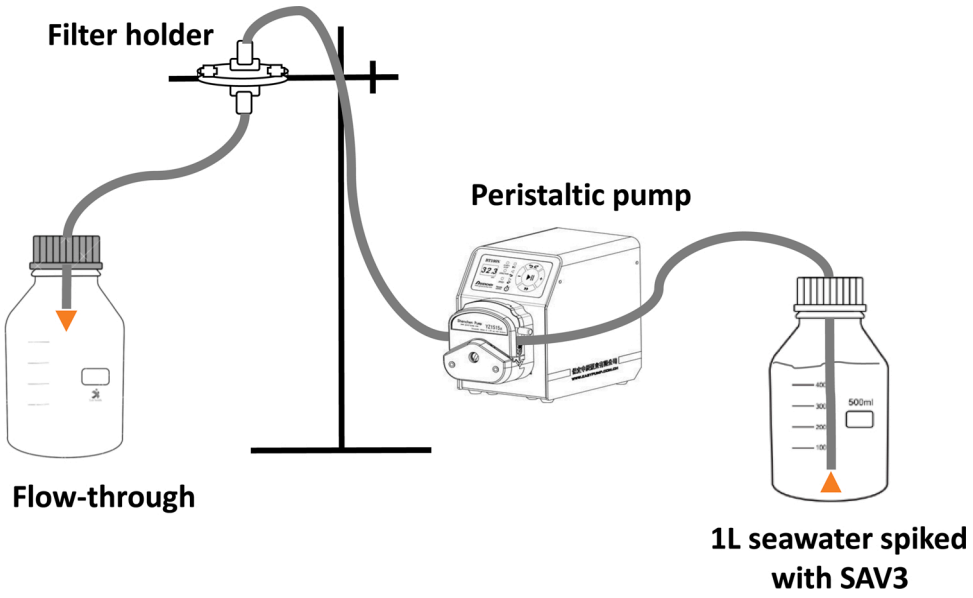

Fig. 1. Schematic diagram of seawater concentration setup.

virus copy numbers in the samples and spike, respectively, *E* is the amplification efficiency of the SAV3 RT-qPCR (104 % = 1.04) and Cq is the cycle quantification.

2.8. Reverse transcription droplet digital PCR (RT-ddPCR)

The primers and probe used for the RT-qPCR were also used for the RT-ddPCR assay. The RT-ddPCR was performed in 20 µL volumes using the One-step RT-ddPCR Advanced Kit for Probe (Bio-Rad), as described by the manufacturer, with 1.8 µL of RNA, and primers and probe at final concentrations of 900 nM and 250 nM, respectively. Droplets were generated on a QX-200 droplet generator (Bio-Rad), before PCR was performed on a T100 instrument, using the following thermal conditions: 60℃ for 60 min, 95℃ for 10 min, followed by 45 cycles of 95℃ for 30 s, 60℃ for 1 min, and a final 98℃ for 10 min. The RT-ddPCR plate was analysed in a QX200™ Droplet Reader (Bio-Rad, USA) using QuantaSoft™ software (Bio-Rad, USA), which was also used for data analysis. The threshold for distinguishing positive from negative droplets was determined manually, as the intensity in relative fluorescence units (RFU) above which no droplet signal would be expected in the non-template controls (NTC). Samples were considered positive for the marker if they contained three or more positive droplets.

2.9. Statistical analysis

All statistical analyses were performed using graph-pad prism 8.3.0. First data was checked for normality, and they were normally distributed. A two-way ANOVA was used to compare differences in SAV3 recovery between the RT-ddPCR and RT-qPCR assays from two-fold serial dilutions (1:1 to 1: 512). A difference of $p < 0.05$ was considered statistically significant. Fishers Least Significant Difference test was used to compare dilutions within the group and between the two groups, and no correction for multiple comparison was made.

3. Results

3.1. Evaluation of filters and buffers for virus recovery

Two different types of membrane filters (one positively and one negatively charged) and four different buffers (buffer 1–4) were evaluated for their capacity to concentrate and elute SAV3 from 1 L seawater samples. The MF- filter/buffer 1 and MD + filter/buffer 1 methods produced the best SAV3 recoveries from natural seawater with 39.5 ± 1.8 % and 19.0 ± 0.1 % (mean ± standard deviation), when the samples were analysed by RT-ddPCR (Table 1). The virus recovery was higher from natural seawater, compared to artificial seawater, when either the MF- filter/buffer 1 or the MD + filter/buffer 1 method was applied. Overall, the highest SAV3 recovery of 39.5 ± 1.8 % was achieved from natural seawater by RT-ddPCR, using the MF- filter/buffer 1

Table 1
Recovery rate (%) of SAV3 in spiked artificial and natural seawater. One litre of seawater was spiked with 1.62×10^7 copies of SAV3 and concentrated, using a negatively charged (MF-) or a positively charged (MD+) filter. Different buffers were used for elution, and SAV3 was quantified with RT-qPCR and RT-ddPCR. SAV3 recovery is presented as mean ± standard deviation from two biological replicates. ND = Not detected.

Membrane	Sample type	Elution buffer	SAV3 recovery (%)	
			RT-qPCR	RT-ddPCR
MF negatively charged filter	Artificial seawater	NucliSENS® lysis buffer	23.7 ± 6.7	38.2 ± 2.9
		1m M NaOH pH 9.0	0.4 ± 0.2	0.4 ± 0.1
		L-15 + 2% FBS pH 9.0	0.4 ± 0.1	0.3 ± 0.1
		L-15 + 2% FBS	0.2 ± 0.1	0.2 ± 0.1
	Natural seawater	NucliSENS® lysis buffer	25.9 ± 5.7	39.5 ± 1.8
		1m M NaOH pH 9.0	1.3 ± 0.4	3.7 ± 2.6
		L-15 + 2% FBS pH 9.0	0.8 ± 0.4	1.7 ± 1.4
		L-15 + 2% FBS	0.7 ± 0.2	1.6 ± 0.3
MD positively charged filter	Artificial seawater	NucliSENS® lysis buffer	6.2 ± 2.5	7.8 ± 4.2
		1m M NaOH pH 9.0	ND	ND
		L-15 + 2% FBS pH 9.0	ND	ND
		L-15 + 2% FBS	ND	ND
	Natural seawater	NucliSENS® lysis buffer	13.3 ± 3.8	19.0 ± 0.1
		1m M NaOH pH 9.0	ND	ND
		L-15 + 2% FBS pH 9.0	ND	ND
		L-15 + 2% FBS	ND	0.3 ± 0.4

method. Furthermore, all tests using buffers 2, 3 and 4 resulted in no or very low recoveries (Table 1).

3.2. The MF-filter/buffer 1 concentration method

Given that our initial experiments showed the MF-filter/buffer 1 method gave the best virus recovery, when 1.62×10^7 SAV3 copies were used to spike 1 L of natural seawater (Table 1), the MF-filter was further evaluated. A two-fold serial dilution (1:1 to 1: 512) was prepared from the SAV3 stock and used to assess the MF-filter capacity for concentration of SAV3 in natural seawater. The results showed the SAV3 recoveries with the MF-filter/buffer 1 method from 1:1 dilution of the dilution series (23.1 ± 5.3 % and 43.1 ± 7.1 %) were similar to the recoveries from our initial experiment (25.9 ± 5.7 % and 39.5 ± 1.8 %) in both RT-qPCR and RT-ddPCR, respectively. Furthermore, results from both RT-ddPCR and RT-qPCR assays of the 2-fold serial dilution (1:2 to 1: 512) of the SAV3 spiked seawater, revealed relatively more SAV3 was recovered from higher dilutions (Table 2). Comparison of results from dilution per group (1:1 to 1: 512), revealed statistically significant increase in SAV3 recovery by RT-qPCR *($p < 0.04$ to 0.0001)*. Similarly, results from dilution per group (1:1 to 1: 512) for RT-ddPCR, also showed statistical increase in SAV3 recovery *($p < 0.04$ to $p < 0.001$)* (Table 2).

3.3. LOD and LOQ of SAV3 in 1 L natural seawater concentrated by the MF-/buffer 1 method

Eleven five-fold dilutions of SAV3 were prepared and added to the natural seawater. The RNA extracted from the natural seawater concentrate was analysed by RT-qPCR and RT-ddPCR to the lowest amount of SAV3 particles detected. As shown in Table 3, SAV3 was detected by both methods from the tested dilutions, except for $1:5^8$ to $1:5^{11}$ dilutions. The LOQ and LOD of RT-ddPCR were estimated to be 5.18×10^3 and 2.0×10^2 SAV3 copies/L of natural seawater respectively (n = 2), while the LOD for RT-qPCR was estimated to be 42 SAV3 copies/L of natural seawater (n = 1); where n is the number of biological replicates.

4. Discussion

The present study demonstrates that *Salmonid alphavirus* spiked in artificial and natural seawater can be recovered after concentration by filtration of water samples. The results showed that the negatively charged MF- filter in general provided better virus recovery than the positively charged MD + filter, and when NucliSENS® lysis buffer (buffer 1) was used to elute SAV RNA from the filter. However, the use of MD + filter in combination with buffer 1 also resulted in fairly good virus recoveries. Thus, the common denominator is that the buffer 1 has the decisive impact in the higher virus recovery, rather than the type of filter used for concentration. Forty percent SAV3 recovery from natural seawater with the MF-/buffer 1 method using RT-ddPCR, indicates a potential for the method to be used for SAV3 recovery under field conditions with farmed salmonids. The method can potentially co-concentrate other viruses well, hence the MF-/buffer 1 method of concentration and elution steps described here may enable accurate assessment of the viruses in seawater samples.

The MF- filter has been used in quantifying poliovirus from 1 L seawater (Katayama et al., 2002). The method required an acidification step to remove cations and promote elution (Fong and Lipp, 2005; Lukasik et al., 2000; SOBSEY, 1995; Sobsey et al., 1973). In the present study, the method used by Katayama and others was modified by directly lysing the adsorbed SAV3 with buffer 1. This modification was introduced in the method development for this study in order to circumvent the laborious acidification step, and to maximize virus recovery during the concentration process.

Previous studies have found that preconditioning of seawater samples with salts facilitates electrostatic adsorption of virus to MF-negative filters (Katayama et al., 2002). Enteric viruses, like polio virus, are negatively charged in water and thus requires cations in the water in order to be linked to a negatively charged membrane.(Katayama et al., 2002; Pallin et al., 1997). The protein capsid of viruses typically contains ionisable amino acids, such as glutamic acid, aspartic acid, histidine, and tyrosine (Gerba, 1984). Like many organic chemicals, these individual carboxyl and amino groups, depending on the pH of the surrounding environment, can gain or lose a proton, giving the capsid a net electrical charge. In this study, preconditioning of the filters was not performed, and it is unclear what kind of interaction was involved in the adsorption of SAV3. However, given that SAV3 is positively charged in seawater, and pH in the water is below the SAV isoelectric point (PI, 9.95) (Pickett et al., 2012), it is plausible for SAV to adsorb to a negatively charged filter, without the need for preconditioning.

The positively charged MD + filter with buffer 1 also facilitates adsorption of SAV from seawater. In this case, the presence of cations and anions in the seawater may have strengthened hydrophobic

Table 2
Recovery rate (%) from a 2-fold serial dilution of SAV3 spiked into seawater. One litre of seawater was spiked with 2-fold dilution series of 1.62×10^7 copies of SAV3 and concentrated using MF-/buffer 1 concentration method. SAV3 recovery using RT-qPCR and RT-ddPCR is presented as mean ± standard deviation from four biological replicates. Two-way ANOVA was used to compare differences in SAV3 recovery between RT-ddPCR and RT-qPCR assays from two-fold serial dilutions.

SAV3 dilutions (copy number)	SAV3 recovery (%)	
	RT-qPCR	RT-ddPCR
1:1 (1.62×10^7)	23.1 ± 5.3	43.1 ± 7.1
1:2 (8.10×10^6)	37.3 ± 4.2	55.8 ± 1.6
1:4 (4.05×10^6)	41.3 ± 6.3	60.6 ± 3.3
1:8 (2.03×10^6)	40.7 ± 4.8*	66.0 ± 5.1*
1:16 (1.01×10^6)	39.6 ± 5.7**	68.6 ± 8.3**
1:32 (5.06×10^5)	57.2 ± 18.0	63.5 ± 9.1
1:64 (2.53×10^5)	56.1 ± 14.2	70.2 ± 4.7
1:128 (1.27×10^5)	46.6 ± 20.6	65.6 ± 2.9
1:256 (6.33×10^4)	48.8 ± 12.5***	78.0 ± 15.2***
1:512 (3.16×10^4)	62.2 ± 15.9	64.0 ± 22.7

Note: *$p < 0.04$, **$p < 0.01$ and ***$p < 0.02$ signs represent the levels of significance.

Table 3
Estimation of the lower limit of detection (LOD), the limit of quantification (LOQ) and the recovery rate (%) of SAV3 in 1 L spiked seawater. One litre of seawater was spiked with 5-fold dilution series (1:1 to 1: 5^{11}) of 1.62×10^7 copies of SAV3 and concentrated using the MF-/buffer 1 concentration method. The LOD for RT-qPCR and RT-ddPCR was given by the lowest amount of SAV3 giving a positive result in the seawater sample, while the RT-ddPCR LOQ was calculated from the highest dilution giving results with a low SD. SAV3 recovery for RT-ddPCR is presented as mean ± standard deviation from two biological replicates. For RT-qPCR, the recovery from one dilution series is given.

SAV3 dilutions (copy number)	SAV3 recovery (%)	
	RT-qPCR	RT-ddPCR
1:1 (1.62×10^7)	30.8	34.4 ± 9.6
1:5 (3.24×10^6)	38.9	37.3 ± 11.0
$1:5^2$ (6.48×10^5)	38.5	40.3 ± 9.5
$1:5^3$ (1.30×10^5)	34.1	36.2 ± 10.0
$1:5^4$ (2.59×10^4)	28.8	37.5 ± 6.2
$1:5^5$ (5.18×10^3)†	42.4	39.9 ± 4.5
$1:5^6$ (1.04×10^3)	28.9	47.1 ± 32.2
$1:5^7$ (207)‡	43.3	124.4 ± 120.1
$1:5^8$ (42)*	32.1	ND
$1:5^9$ (8)	ND	ND
$1:5^{10}$ (1.6)	ND	ND
$1:5^{11}$ (0)	ND	ND

Note: † represents (LOQ) for RT-ddPCR, ‡ represents LOD for RT-ddPCR while * represents LOD for RT-qPCR. ND = Not detected.

reactions between SAV3 and filter, which led to SAV3 adsorption. Similar findings have been reported previously (Lukasik et al., 2000).

In addition, negatively charged filters have been used for recovery of solid-associated viruses from effluent, raw sewage and sludge samples (Agency, 1984; UKWIR, 2000; Wyn-Jones and Sellwood, 2001). In the current study, both artificial and natural seawater were used, and more virus was concentrated from natural seawater that contained organic matters compared to the artificial seawater, which correlates with previous reports (Agency, 1984; UKWIR, 2000; Wyn-Jones and Sellwood, 2001).

Inhibitions may cause major problems when detecting or quantifying RNA virus in seawater concentrates by RT-qPCR methods (Gibson et al., 2012; Girones et al., 2010). In an effort to increase detection sensitivity, the use of robust extraction and amplification methods is recommended, as organic matter that may interfere with the enzymes used for amplification are co-concentrated with viral particles. In the present study, RT-ddPCR was applied in order to overcome the problem of inhibition (Rački et al., 2014). We assessed the MF-/buffer 1 method capacity for concentration of SAV3 in seawater from 2-fold serial dilutions. It appears that relatively more SAV3 was recovered from the higher dilutions compared to the lower dilutions (Table 2), which may suggest that MF-filter may be more efficient at concentrating at higher dilutions of SAV3. This is interesting, as virus content in seawater is usually low, and a method that enhances recovery from environmental samples would be important for risk assessment and surveillance. Furthermore, we compared SAV3 recovery as quantified by RT-qPCR to RT-ddPCR. At lower dilution (1:1 to 1:4), there was no significant differences in SAV3 recoveries between RT-qPCR and RT-ddPCR (Table 2). However, at higher dilutions (from 1:8 to 1:16; $p < 0.04$, 0.01 and at 1: 256; $p < 0.01$), significant differences between SAV3 recovery from RT-ddPCR and RT-qPCR was observed. The RT-ddPCR and RT-PCR gave highly divergent LOD data, which is probably due to few biological replicates. More SAV3 was detected with RT-ddPCR compared to RT-qPCR. Thus, RT-ddPCR appears to be more sensitive than RT-qPCR in accordance with previous reports (Burns et al., 2010; Morisset et al., 2013). RT-ddPCR therefore is a useful alternative method for detection and quantification of SAV3 in seawater using the method with the negatively charged filter with NucliSENS® lysis buffer, and the assay may be extended to quantification of other seawater fish viruses.

In summary, the method developed in this study is capable of detecting *Salmonid alphavirus* subtype 3 from natural seawater samples. We hypothesize that the concentration method developed in this study has the potential to be applied for disease surveillance purposes in farmed populations of salmonid fish in seawater, and may be applied to detect other fish pathogenic viruses in water (e.g., *Infectious salmon anaemia virus*, *Salmon gill poxvirus*, *Piscine orthoreovirus*).

CRediT authorship contribution statement

Simon Chioma Weli: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Lisa-Victoria Bernhardt:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing, Visualization. **Lars Qviller:** Writing - review & editing, Supervision. **Mette Myrmel:** Validation, Formal analysis, Data curation, Writing - review & editing, Visualization, Supervision. **Atle Lillehaug:** Conceptualization, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors have no competing interests.

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