Paper III

Sensitive and specific detection of *Salmonid alphavirus* using real-time PCR (TaqMan®)

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Abstract

Pancreas disease is responsible for major economic losses in the European salmonid farming industry. It was previously believed that a single subtype (salmon pancreas disease virus) of the virus species *Salmonid alphavirus* (SAV) was responsible for all outbreaks of pancreas disease in the UK and Norway. However, the recent discovery that pancreas disease in Norway is caused by a new and distinct subtype of salmonid alphavirus, exclusively found in Norway, has advocated the need for better diagnostic tools. In the present paper, three real-time PCR assays for all known subtypes of salmonid alphavirus have been developed; the Q_nsP1 assay is a broad-spectrum one that detects RNA from all subtypes, the Q_SPDV assay specifically detects the salmon pancreas disease virus subtype, and the Q_NSAV assay only detects the new Norwegian salmonid alphavirus subtype.

The results demonstrated the assays to be highly sensitive and specific, detecting <0.1 $TCID_{50}$ of virus stocks. Regression analysis and standard curves calculated from the C_t-values from 10-fold serial dilutions of virus stocks showed that the assays were highly reproducible over a wide range of RNA input. Thirty-nine field samples were tested in triplicate and compared with traditional RT-PCR. Overall, the real-time assays detected 35 positive compared to 29 positives in standard RT-PCR, and were thus shown to be more sensitive for detecting salmonid alphaviruses in field samples.
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Keywords: *Salmonid alphavirus*; Real-time PCR; Virus detection

1. Introduction

Pancreas disease in farmed salmonids is commonly associated with infections by *Salmonid alphavirus* (SAV). The disease occurs mainly in Atlantic salmon in their first or second year at sea, and diseased fish are often lethargic, with abnormal swimming behaviour. Histopathological lesions in association with pancreas disease always include various degrees of heart and skeletal muscle myopathy. Acute and chronic pancreatic lesions in exocrine pancreatic tissue may also be present in diseased fish (McLoughlin et al., 2002). The virus responsible for pancreas disease in Ireland and Scotland have been isolated and identified as an alphavirus, and the name salmon pancreas disease virus (SPDV) was suggested (Nelson et al., 1995; Welsh et al.,

2000; Weston et al., 1999). Because pancreas disease affected fish from Norway show similar clinical symptoms and gross pathology, it has been of the common opinion that pancreas disease in the British Isles and Norway is caused by the same virus. However, Hodneland et al. (2005) recently showed that pancreas disease from Atlantic salmon and rainbow trout in Norway is in fact caused by a different and distinct virus subtype, and named it Norwegian salmonid alphavirus (NSAV). Together with the sleeping disease virus (SDV) (Boucher and Baudin Laurencin, 1994; Branson, 2002; Castric et al., 1997; Graham et al., 2003b; Villoing et al., 2000a) and salmon pancreas disease virus, the Norwegian salmonid alphavirus is included in the species *Salmonid alphavirus* in the genus Alphavirus of the family Togaviridae. All three salmonid alphavirus subtypes have a genomic organization characteristic to the Alphaviruses; with a positive-sense, single stranded genome of approximately 11.8 kb size. The 5′-terminal end codes for the four non-structural proteins (nsP1–nsP4) essential for virus replication, whereas the 3′-terminal comprises the genes for the structural proteins E1–E3,

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capsid and 6K. Nucleotide sequence comparisons have shown that Norwegian salmonid alphavirus, salmon pancreas disease virus and sleeping disease virus are approximately equal in evolutionary distance to each other, with differences ranging from 91.6 to 92.9% (Hodneland et al., 2005).

Previously, the diagnosis of pancreas disease and sleeping disease was based on clinical sign in combination with histopathological findings. Detection of antibodies or virus isolation in fish cells may also be used to verify the aetiology of the disease (Graham et al., 2003a; Jewhurst et al., 2004). However, the presence of virus-specific antibodies does not provide any information about the viraemic status of an infected fish, and considering the high percentage similarity of the structural proteins among the salmonid alphaviruses, the potential for cross-reactivity of serological assays is high. Furthermore, it is not possible to unequivocally distinguish subtypes of salmonid alphavirus in cell-cultures. As a result of the relative insensitivity of non-molecular detection methods, molecular methods such as RT-PCR based techniques have been developed for a number of fish RNA viruses, and have been demonstrated successfully to increase the detection rate. Villoing et al. (2000b) presented a two-step RT-PCR assay for detection of sleeping disease virus RNA in naturally infected salmonids, which also proved useful for amplification of salmon pancreas disease virus in experimentally infected fish. However, the RT-PCR could not discriminate between the two subtypes without further sequencing studies.

With the discovery of the third salmonid alphavirus subtype and the distinct geographical distribution of at least salmon pancreas disease virus and Norwegian salmonid alphavirus, the ability to distinguish between types or strains of virus that may have distinct biological properties is important for both national and international management and control of the disease. At present, existing methods are not sufficient to rapidly distinguish between the different pheno-/genotypes, and the development of a more powerful diagnostic assay for direct identification of salmonid alphavirus subtypes, with respect to sensitivity, specificity and speed will be useful. The real-time PCR technology is now used commonly for detection and quantification of many viruses (Mackay et al., 2002; Niesters, 2001; Niesters, 2002), however the only piscine viruses where real-time PCR assays have been developed are piscine nodavirus (Starkey et al., 2004), infectious salmon anemia virus (ISAV) (Munir and Kibenge, 2004) and infectious haematopoietic necrosis virus (IHNV) (Overturf et al., 2001).

The present paper describes the development and validation of real-time PCR assays for the sensitive detection and differentiation of three subtypes of salmonid alphavirus (salmon pancreas disease virus, sleeping disease virus and Norwegian salmonid alphavirus) using the TaqMan® probe chemistry. By developing these assays it is now possible to screen rapidly for all known salmonid alphavirus subtypes without the need for prior isolation and culture, or time-consuming post-PCR steps. The advantage of using real-time PCR for detection of salmonid alphaviruses not only saves time and labor, but also has the potential to differentiate and quantitate any subtype of salmonid alphavirus within the host.

2. Material and methods

2.1. Virus stocks and clinical samples

The specificity of the real-time PCR assay was determined using control strains as a RNA source. Cultured virus stocks from three different subtypes of salmonid alphavirus were used as reference templates in the real-time assays: Norwegian salmonid alphavirus isolated from Norwegian salmon suffering from pancreas disease (SavH10/02, Genebank accession no. AY604237), salmon pancreas disease virus from pancreas disease affected salmon from Ireland (F93-125, Genebank accession no: AJ316244), and a sleeping disease virus isolate originating from rainbow trout from France (kindly supplied by Dr. K.E. Christie, Intervet Norbio AS, Bergen, Norway). Virus titers ($TCID_{50}$) was $10^{5.8}$/ml for Norwegian salmonid alphavirus, $10^{7.6}$/ml for salmon pancreas disease virus, and $10^{6.25}$/ml for the sleeping disease virus cell culture. Heart tissues from 39 salmon from various fish farms were collected and tested to evaluate the performance of the real-time PCR assays from field samples. The fish in the salmon farms were diagnosed, or suspected to suffer from pancreatic disease.

2.2. RNA extraction

RNA extraction from both infected cell cultures and tissues was performed as described by Devold et al. (2000). The purity of the RNA was evaluated by measuring the absorbance ratio at 260/280 nm (optimal 1.8–2.0), and RNA quality was checked on ethidium bromide-stained agarose (1%) gel using UV illumination. RNA from tissue samples was dissolved in RNAse free water at a working concentration of 100 ng/ul.

2.3. Standard RT-PCR

Standard RT-PCR assays were performed by incubating 2 ul of dissolved total RNA with 1.0 ul (1 ug/ul) random hexamer pd(N)6 primer and 7.0 ul ddH$_2$O at 70 °C for 5 min and placed on ice. The RT-reaction was carried out at 37 °C for 60 min with 10 U Rnasin, 5.0 μl 5 × RT-buffer, 3.0 U M-MLV-reverse transcriptase, 1.25 μl DTT (200 mM), 2.5 μl dNTP (10 mM). The PCR was performed in a 25 ul reaction volume containing 2.0 μl cDNA template, 2.5 μl 10 × Taq buffer, 1.0 μl (10uM) of each PCR primer (Table 1), 2.0 μl (10 mM) dNTP mix, 0.1 μl (5 U/ul) Taq DNA polymerase and 16.4 μl ddH$_2$O. The PCR profile was as follows: one cycle at 95 °C in 3 min; then 40 cycles at 94 °C for 30 s; 55 °C for 45 s; and 72 °C for 90 s; followed by one cycle at 72 °C for 10 min. The amplification and cDNA synthesis were performed in GeneAmp PCR System 9700 (Perkin-Elmer). PCR products were visualized on an ethidium bromide-stained agarose (1%) gel using UV illumination.

2.4. Primers and probes

TaqMan PCR primers and probes were designed according to standard cycling conditions using the PrimerExpress software package (PE Applied Biosystems), and were derived from an

Table 1
Sequences and positions of primers and probes used for real-time assays and conventional RT-PCR

Oligonucleotide	Sequence	Amplicon length	Position	Position no.
Q_SPDV F primer	5′-ACAGTGAAATTCGACAAGAAATGC-3′	68	9555–9578	NC003930
R primer	5′-TGGGAGTCGCTGGTGAAAGT-3′		9603–9622	
Probe	FAM-5′-AGAGCGCTGACTCGGCAACCGT-3′-MGB		9580–9601	
Q_NSAV F primer	5′-CAGTGAAATTCGATAAGAAGTGCAA-3′	67	9431–9455	AY604235
R primer	5′-TGGGAGTCGCTGGTAAAGGT-3′		9478–9497	
Probe	FAM-5′-AGCGCTGCCCAAGCGACCG -3′-MGB		9457–9475	
Q_nsP1 F primer	5′-CCGGCCCTGAACCAGTT-3′	107	17–33	AY604235
R primer	5′-GTAGCCAAGTGGGAGAAAGCT-3′		54–69	
Probe	FAM-5′-CTGGCCACCACTTCGA-3′-MGB		103–123	
2234 F primer	5′-CGGGTGAAACATCTCTGCG-3′	539	9014–9032	AY604235
2767 R primer	5′-CTTGCCCTGGGTGATACTGG-3′		9533–9552	
F4 primer	5′-AGCGACTCCCAGACGTTTACG-3′	899	9487–9507	AY604235
R1 primer	5′-CGGTTTATCACTGCTTCGTACGA-3′		10363–10385	

alignment of available sleeping disease virus, salmon pancreas disease virus and Norwegian salmonid alphavirus sequences. Primer pairs and probes that demonstrated 100% homology to their respective sequences, and at the same time discriminated between the subtypes were selected (Fig. 1). Two of these real-time PCR assays, Q_SPDV and Q_NSAV, amplify an identical region in the E2 gene of salmon pancreas disease virus and Norwegian salmonid alphavirus, respectively. A common primer
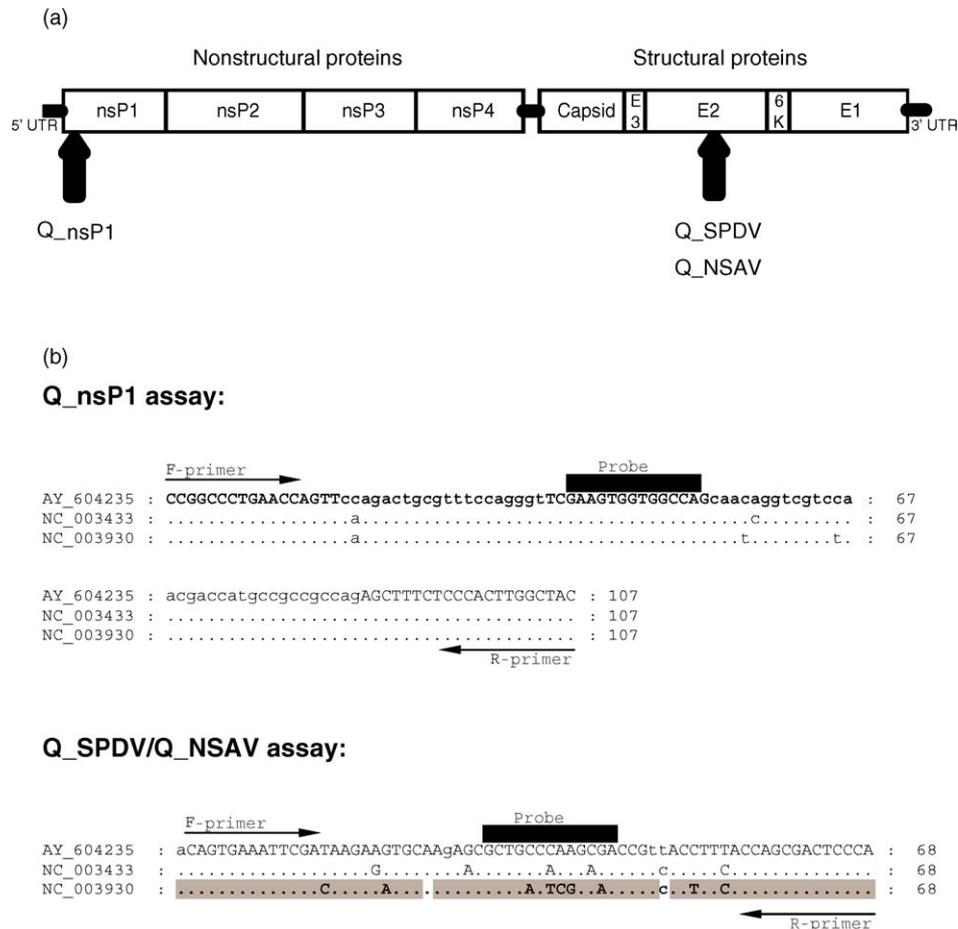

Fig. 1. (a) Orientation of primers and probes in relative position to the genome organization of salmonid alphaviruses. (b) Sequence comparisons of the three salmonid alphavirus subtypes, Norwegian salmonid alphavirus (AY604235), sleeping disease virus (NC003433) and salmon pancreas disease virus (NC003930) in the targeted regions for Q_nsP1, Q_SPDV and Q_NSAV assay. In the Q_nsP1 assay the common primers and probe for all salmonid alphaviruses are indicated by bold letters. Primers and probe used in the Q_SPDV assay are denoted by shaded, bold letters in the sequence for NC_003930. The Q_NSAV assay uses primers and probe sequences shown as letters in bold in the AY_604235 sequence.

pair and probe for all available salmonid alphavirus sequences was designed, and this real-time assay (Q_nsP1) amplifies a region in the 5′ end of the nsP1-gene. All primers and probes were obtained from PE Applied Biosystems. The PCR primers 2234F and 2767R were applied in the standard RT-PCR reaction, and amplify a 539 bp fragment of the E2 gene. This primer pair has been used previously and routinely for detection of salmonid alphaviruses in the laboratory. In addition, the primer pair F4-R1 (Hodneland et al., 2005) was used for amplification of infected tissue samples with subsequent sequencing in order to verify the identity of the virus species detected in the real-time assays. Details on all primers and probes used in this study are summarized in Table 1.

2.5. Real-time PCR

TaqMan assays were performed with 2 ul of cDNA (template), 900 nM of each primer, and 200 nM of probe in a total volume of 25 ul by using the TaqMan Universal PCR Master Mix w/AmpErase® UNG (PE Applied Biosystems). Amplification (45 cycles) and fluorescence detection were performed with the ABI Prism 7000 sequence detection system instrument as recommended by the manufacturer (PE Applied Biosystems). Each sample was tested in triplicate. Samples were considered positive when the fluorescence signal increased above the threshold cycle (C_t), and if the C_t value was ≤ 37.5. The threshold value for all tests was fixed at 0.2, which was within the linear phase of the exponential amplification and above background fluorescence noise. The cut-off C_t value at 37.5 was set based on two-fold dilutions (30 replicates) of a viral cDNA-stock, and denotes the mean C_t value in the highest dilution for which all 30 replicates were positive (results not shown).

2.6. Plasmid preparation

PCR fragments encompassing their respective probing regions of the Q_SPDV, Q_NSAV and Q_nsP1-assays for all three virus strains were cloned into the pCR®4-TOPO® vector (Invitrogen) under conditions recommended by the supplier. Plasmids were linearized and the template (dsDNA) copy number was calculated by UV spectroscopy at an optical density of 260 nm. Plasmid preparations were diluted to a final stock concentration of 5×10^7 copies/ul.

2.7. Standard curves and detection limits

For each real-time PCR assay a standard curve was generated using a 10-fold serial dilutions (three parallels) of the virus stock RNA's. An aliquot of 100 ul of each viral dilution was extracted as described above. Regression analysis, standard curve slopes s (C_t versus log quantity), and amplification efficiencies E ($E = [10^{1/(-\text{slope})}] - 1$) were calculated. If the amplification efficiency of the reaction is ideal, or 1, the PCR product concentration doubles during every cycle within the exponential phase of the reaction. The accepted amplification efficiency range is 0.9–1.1. A similar approach was used to make standard curves from salmonid alphavirus infected fish tissue (heart

ventricle) from field samples (5-fold serial dilutions). This test was done in order to check for any limitations of the assays from field samples compared to virus samples from CHSE-214 cell cultures, such as the presence of endogenous inhibitors in template RNA preparations.

Data from the standard curve estimations above were also used to determine the limit of detection of the real-time assays. The detection limit describes the lowest amount of a template that can be detected under optimal conditions, and was defined as the highest dilution factor for which all samples (triplicate) were positive in the specific real-time assay. This was done by direct comparison of observed C_t values with the respective serial dilutions of infectious virus titers (TCID$_{50}$). Establishing the relationship between the real-time PCR detection limits and corresponding dilution of the TCID$_{50}$ virus stocks will give an indication of the detection performance of the two different methods. Similarly, detection limits for each real-time PCR assay was determined for serial dilutions of known amounts of linearized plasmid (template copies). This latter test was performed in order to check the efficiency of the PCR in the real-time assays.

3. Results

3.1. Specificity

The Q_nsP1 TaqMan assay was designed specifically to detect all known salmonid alphaviruses, whereas the Q_SPDV and Q_NSAV assays were designed to discriminate between salmon pancreas disease virus and Norwegian salmonid alphavirus, respectively. The location of primers and probes for all three assays were chosen carefully for distinguishing salmonid alphaviruses from other viruses. No fluorescent signal for the three assays was generated when RNA samples from tissues infected with ISAV, IPNV, and Nodavirus was tested (data not shown). The specificity of the different probes on salmonid alphavirus was assessed by testing the viral stocks of Norwegian salmonid alphavirus (Norway; SavH10/02), salmon pancreas disease virus (Ireland; F93–125), and sleeping disease virus (France). All three salmonid alphavirus strains were detected using the Q_nsP1 assay, whereas the Q_SPDV assay only detected the F93–125 samples and the Q_NSAV was positive for only for the SavH10/02 strain. Real-time PCR on all combinations of mixes of cDNA from the three virus strains did not affect the specificity of the different assays (data not shown). Furthermore, real-time PCR assays on the plasmid preparations did not produce any false negatives or positives. Standard RT_PCR on virus RNA using the F4-R1 primers produced amplicons of correct length, and subsequent sequencing of the PCR-products identified the samples as the correct virus species.

Total RNA preparations of heart tissue samples from 39 Atlantic salmon collected from various fish farms were screened (Table 2). The Q_nsP1 assay was first applied to check for presence of salmonid alphavirus in the samples. If the sample was found to be positive, the presence of salmon pancreatic disease virus or Norwegian salmonid alphavirus subtype were checked specifically by using the Q_SPDV and Q_NSAV real-

Table 2
Comparison of salmonid alphavirus detection from fish samples by real-time PCR assays and standard RT-PCR

Fish no.	Sample (location and year of collection)	TaqMan assay			Standard RT-PCR
		Q_nsP1	Q_SPDV	Q_NSAV	
1	HO (97)_1	31.40	–	32.69	3/3
2	HO (97)_2	36.20	–	37.74	0/3
3	HO (97)_3	–	–	–	0/3
4	HO (97)_4	–	–	–	0/3
5	HO (97)_5	–	–	–	0/3
6	HO (97)_6	29.70	–	31.19	3/3
7	HO (99)_1	24.90	–	26.39	3/3
8	HO (99)_2	30.10	–	31.63	3/3
9	SF (03)_1	32.02	–	33.50	3/3
10	SF (03)_2	32.30	–	33.72	3/3
11	SF (03)_3	31.60	–	33.12	3/3
12	SF (03)_4	29.22	–	30.62	3/3
13	SF (03)_5	19.77	–	21.33	3/3
14	SF (03)_6	33.59	–	35.20	1/3
15	SF (03)_7	34.87	–	36.33	0/3
16	SF (03)_8	28.88	–	30.36	3/3
17	SF (03)_9	35.10	–	36.68	0/3
18	SF(03)_10	34.19	–	35.69	3/3
19	HO (04)_1	31.39	–	32.82	3/3
20	HO (04)_2	27.19	–	28.65	3/3
21	HO (04)_3	23.63	–	25.10	3/3
22	HO (04)_4	19.80	–	21.23	3/3
23	HO (04)_5	33.37	–	34.91	2/3
24	HO (04)_6	28.63	–	30.13	3/3
25	HO (04)_7	23.25	–	24.59	3/3
26	HO (04)_8	19.27	–	20.72	3/3
27	HO (04)_9	22.60	–	24.11	3/3
28	HO (04)_10	30.01	–	31.61	3/3
29	HO (04)_11	33.03	–	34.55	2/3
30	SF (04)_1	–	–	–	0/3
31	SF (04)_2	33.32	–	34.32	2/3
32	SF (04)_3	37.49	–	39.01	0/3
33	SF (04)_4	36.81	–	38.33	0/3
34	SF (04)_5	33.89	–	35.48	1/3
35	SF (04)_6	31.64	–	33.01	3/3
36	SF (04)_7	29.09	–	30.55	3/3
37	SF (04)_8	35.41	–	36.99	0/3
38	SF (04)_9	30.18	–	32.01	3/3
39	SF (04)_10	34.81	–	36.33	2/3
No. of fish positive		35	0	35	29
No. of fish negative		4	39	4	10

time PCR. Thirty-five cases of Norwegian salmonid alphavirus were detected with both the Q_nsP1 and Q_NSAV assay. No cases with salmon pancreas disease virus from the samples were detected. Standard RT-PCR detected 29 salmonid alphavirus infected fish, all of these had C_t-values of <34.87 (Q_nsP1 assay). Some fish had very low C_t-values (<20.0) indicating large amount of viral templates in the examined tissue. The six fish which tested negative in the RT-PCR screening but were positive in the Q_nsP1 assay, had C_t-values of 36.20, 34.87, 35.10, 37.49, 36.81 and 35.41. Five of the 29 positive fishes were only positive in one or two of the replicates in the standard RT-PCR (Table 2). The C_t-values (Q_nsP1) from these five fishes ranged from 33.03 to 34.81. To validate the specificity of the TaqMan assays, cDNA from positive fish were checked with PCR with the F4–R1 primer combination, and sequencing showed that all were of the Norwegian salmonid alphavirus subtype.

3.2. Standard curves

The standard curve for the serial dilutions (10-fold) of the virus stocks were calculated for all three real-time PCR assays. The mean slopes for all assays were similar (Fig. 2 and Table 3), and the amplification efficiency (E) indicated near maximum PCR efficacy ($E = 0.965–1.035$). Almost identical standard curve slopes and C_t-values were obtained with dilutions either before RT at the RNA level, or at the cDNA level (data not shown). Furthermore, serial dilutions (5-fold) from infected tissues from field samples produced standard curve slopes and amplification efficiencies similar to the above (Table 3).

3.3. Sensitivity

Assay detection range for the three different real-time PCR assays and standard RT-PCR with the primer pair 2234F–2767R are summarized in Table 4. Each of the real-time assays was evaluated by comparison of the dilution limit C_t-value (C_t) and their respective virus titre. For the standard RT-PCR assay samples were considered positive when a visible band of the correct size was observed on an ethidium bromide-stained agarose gel. When tested on the salmon pancreas disease virus—virus stock the sensitivity of Q_nsP1 assay was slightly higher than the Q_SPDV assay, although not statistically significant. How-

Table 3
Summary of standard curve slopes (s), regression coefficients (R^2) and amplification efficiencies (E) for the three real-time PCR assays from viral stocks and fish tissue samples

Salmonid alphavirus subtype	Origin	Q_nsP1			Q_SPDV			Q_NSAV		
		s	R^2	E	s	R^2	E	s	R^2	E
Sleeping disease virus[a]	Viral stock	−3.22	0.999	1.004	–	–	–	–	–	
Salmon pancreas disease virus	Viral stock	−3.24	0.998	1.035	−3.37	0.999	0.980	–	–	–
	Fish sample	−3.30	0.990	1.009	−3.41	0.989	0.965	–	–	–
Norwegian salmonid alphavirus	Viral stock	−3.37	0.997	0.980	–	–	–	−3.31	0.996	1.005
	Fish sample	−3.39	0.991	0.972	–	–	–	−3.32	0.999	1.001

[a] Only virus samples from CHSE-214 cell cultures were tested.

Q_nsP1 assay

■ Norwegian salmonid alphavirus ▲ Salmon pancreas diseasevirus ◆ Sleeping disease virus

Q_SPDV assay

▲ Salmon pancreas disease virus

Q_NSAV assay
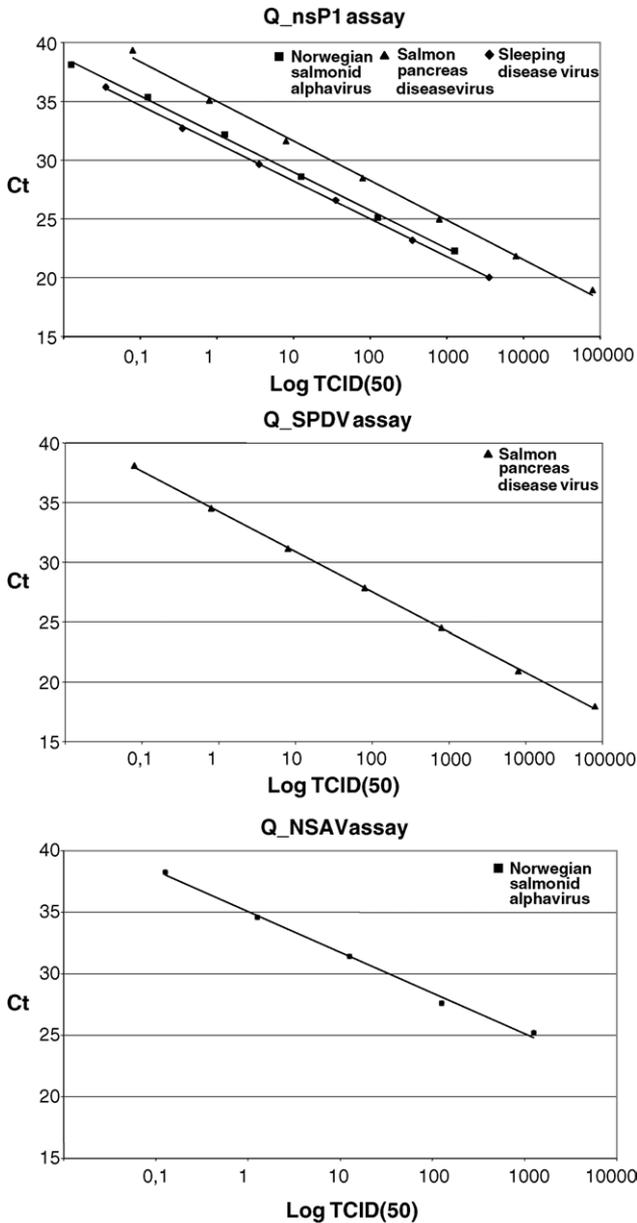
■ Norwegian salmonid alphavirus

Fig. 2. Regression analysis and standard curves for the Q_nsP1, Q_SPDV and Q_NSAV assay from virus infected CHSE-214 cells supernatant. The starting amount (undiluted sample) for the virus stocks were $10^{5.8}$/ml, $10^{7.5}$/ml and $10^{6.25}$/ml for the Norwegian salmonid alphavirus, salmon pancreas disease virus and sleeping disease virus, respectively. Ten-fold serial dilutions of each virus stock were tested, and the mean C_t-values for each triplicate were plotted against the $TCID_{50}$ dilution. Regression analysis and standard curves for the Q_nsP1, Q_SPDV and Q_NSAV assay from virus infected CHSE-214 cells supernatant. The starting amount (undiluted sample) for the virus stocks were $10^{5.8}$/ml, $10^{7.5}$/ml and $10^{6.25}$/ml for the Norwegian salmonid alphavirus, salmon pancreas disease virus and sleeping disease virus, respectively. Ten-fold serial dilutions of each virus stock were tested, and the mean C_t-values for each triplicate were plotted against the $TCID_{50}$ dilution.

ever, the Q_NSAV assay was significantly less sensitive than the Q_nsP1 assay by an average of 2.7 C_t-values irrespective of the dilution factor. Amplification plots of the Q_nsP1 assay showed that dilutions of 10^{-6}, 10^{-5} and 10^{-6} for the salmon pancreas disease virus, sleeping disease virus, and Norwegian salmonid

Table 4
Sensitivity comparisons of the different real-time PCR assays and standard RT-PCR for the detection of salmonid alphaviruses

| Dilution | Q_nsP1 assay | | | | | | Q_SPDV assay | | Q_NSAV assay | | Standard RT-PCR[*] | | |
| | Salmon pancreas disease virus | | Sleeping disease virus | | Norwegian salmonid alphavirus | | Salmon pancreas disease virus | | Norwegian salmonid alphavirus | | Salmon pancreas disease virus | Sleeping disease virus | Norwegian salmonid alphavirus |
	C_t	Result	C_t	Result	C_t	Result	C_t	Result	C_t	Result	Result	Result	Result
Undiluted	18.9	3/3	20.0	3/3	22.3	3/3	18.0	3/3	25.2	3/3	3/3	3/3	3/3
10^{-1}	21.8	3/3	23.2	3/3	25.1	3/3	20.9	3/3	27.6	3/3	3/3	3/3	3/3
10^{-2}	25.0	3/3	26.6	3/3	28.6	3/3	24.6	3/3	31.4	3/3	3/3	3/3	3/3
10^{-3}	28.5	3/3	29.6	3/3	32.2	3/3	27.9	3/3	34.6	3/3	3/3	3/3	3/3
10^{-4}	31.6	3/3	32.7	3/3	35/4	3/3	31.1	3/3	38.3	3/3	3/3	1/3	0/3
10^{-5}	35.1	3/3	36.2	3/3	38.1	3/3	34.5	3/3	–	0/3	0/3	0/3	0/3
10^{-6}	39.3	3/3	–	0/3	42.1	2/3	38.1	3/3	–	0/3	0/3	0/3	0/3
10^{-7}	–	0/3	–	0/3	–	0/3	–	0/3	–	0/3	0/3	0/3	0/3

The $TCID_{50}$ for undiluted virus samples were $10^{7.6}$/ml for salmon pancreas disease virus, $10^{6.25}$/ml for sleeping disease virus, and $10^{5.8}$/ml for the Norwegian salmonid alphavirus from cell culture.

Table 5
Sensitivity comparisons of the different real-time PCR assays on linearized plasmid preparations containing the appropriate target sequence

| Plasmid copies | Q_nsP1 assay | | | | | | Q_SPDV assay | | Q_NSAV assay | |
| | SPDV_P1 | | SDV_P1 | | NSAV_P1 | | SPDV_P2 | | NSAV_P2 | |
	C_t	Result	C_t	Result	C_t	Result	C_t	Result	C_t	Result
10^8	17.42	3/3	16.95	3/3	17.36	3/3	17.12	3/3	19.84	3/3
10^7	20.67	3/3	19.02	3/3	20.42	3/3	20.25	3/3	22.76	3/3
10^6	23.92	3/3	22.30	3/3	22.74	3/3	23.09	3/3	25.51	3/3
10^5	27.25	3/3	26.17	3/3	26.24	3/3	26.84	3/3	29.27	3/3
10^4	31.10	3/3	29.07	3/3	29.79	3/3	30.39	3/3	32.28	3/3
10^3	34.41	3/3	32.26	3/3	33.21	3/3	33.67	3/3	35.69	3/3
10^2	37.26	3/3	35.86	3/3	37.01	3/3	37.09	3/3	39.30	3/3
10^1	39.97	3/3	39.49	3/3	39.55	3/3	39.72	3/3	42.56	2/3
1	41.99	2/3	42.13	1/3	41.12	1/3	43.79	1/3	–	0/3
Slope (s)	−3.29		−3.27		−3.25		−3.31		−3.25	

The plasmid copies refer to the total input of linearized plasmid copies in each reaction.

alphavirus strains, respectively, could be detected. Including all extraction steps, this correlates with a sensitivity of 0.08 $TCID_{50}$ for salmon pancreas disease virus, 0.04 $TCID_{50}$ for sleeping disease virus and 0.01 for Norwegian salmonid alphavirus per reaction.

Similarly, the real-time PCR on the linearized plasmid dilution series also indicated that the Q_NSAV assays was less sensitive than the Q_nsP1 assay. Using the same plasmid dilutions as template there was an average of 2.6 C_t-values higher for the Q_NSAV assay compared with the C_t values for the Q_nsP1 assay.

Overall, the detection limit for Q_NSAV was between 10 and 100 plasmid copies (NSAV_P2), and the Q_SPDV assay detected 1–10 copies of the SPDV_P2 plasmid. Sensitivity test of the Q_nsP1 assay on the plasmids SPDV_P1, SDV_P1 and NSAV_P1 showed that between 1 and 10 plasmid copies could be detected for all three plasmid preparations (Table 5). Regression analysis on the plasmid dilution series demonstrated that all assays were quantitative with standard curve slopes (s) ranging from −3.25 to −3.31.

4. Discussion

Initially, a real-time PCR assay (Q_SPDV) was designed as a less labour consuming and more reliable diagnostic test than previous assays used by the official diagnostic laboratory. Primers and probes were designed to match 100% to the available salmon pancreas disease virus sequences deposited in the Genebank (NC003930, AJ012631). The Q_SPDV assay was tested on a laboratory strain of pancreas disease virus with great success, but when applied on field samples from diseased fish diagnosed as pancreas disease the assay were not able to produce any positive results. Through a series of RT-PCRs and sequencing studies it became clear that the virus causing pancreas disease in Norway is a separate subtype of the salmonid alphavirus species (Hodneland et al., 2005), and that the sequence differences between Norwegian salmonid alphavirus and salmon pancreas disease virus in the targeted Q_SPDV area were sufficiently large to effectively prevent any amplification from Norwe-

gian salmonid alphavirus templates. A new assay was designed which amplifies the same region as in the Q_SPDV assay, but directed specifically against the Norwegian salmonid alphavirus sequence (AY604235). Furthermore, a common assay (Q_nsP1) specific for salmonid alphavirus was made in order to detect any of the three subtypes of the salmonid alphavirus species (salmon pancreas disease virus, Norwegian salmonid alphavirus and sleeping disease virus). In the present study, it is shown that the specificity for the three assays is 100% for all available salmonid alphavirus sequences, and gives no false positives or negatives. The use of AmpErase uracil-N-glycosylase (UNG) in the PCR amplification step also greatly reduces or eliminates the potential source of carry-over contamination of samples which is crucial, at least for diagnostic purposes, in laboratories with large sample through-put (Burkardt, 2000; Hofmann-Lehmann et al., 2000; Pennings et al., 2001; Taggart et al., 2002).

A wide range of virus concentrations was linearly amplified in the Q_nsP1, Q_SPDV and Q-NSAV assays, with a near maximum PCR efficiency for all three (Table 3, Fig. 2). Similarly, plasmid copies ranging from 10^8 to 10 per reaction tested positive with the assays with amplification efficiencies (E-values) near 1.0. The common Q_nsP1 assay developed for all salmonid alphavirus species detected virus concentrations ($TCID_{50}$) from serial dilutions of virus stocks ranging from $10^{4.9}$/reaction for salmon pancreas disease virus, to 0.01/reaction for the Norwegian salmonid alphavirus virus stock. The observed linearity in the wide range of virus titres should allow detection of salmonid alphaviruses from field samples in all stages of the disease in fish; early infection, viremiae with clinical pancreatic disease, or latency/persitent infections.

The Q_SPDV assay was equally sensitive as the Q_nsP1 assay when tested on the same template dilution series, making both assays suitable for further applications on studies on the salmon pancreas disease virus subtype. However, the Q_NSAV assay performed consistently lower than the Q_nsP1 assay when both assays were tested on identical template. This is somewhat surprising since the Q_NSAV assay detects and amplifies the same target region as in the Q_SPDV assay. Amplification and sequencing of this particular Norwegian salmonid alphavirus

genome stretch shows that the Q_NSAV primer and probes match 100% to the Norwegian salmonid alphavirus genome, and that mismatches cannot account for the lowered sensitivity of Q_NSAV. The differences between the primers and probes for the Q_SPDV and Q_NSAV are shown in Fig. 1(b), and involve two substitutions in both the forward and reverse primer, and five substitutions in the probe sequence. These substitutions do not alter the G + C composition or introduce any hairpin structures or stretches of identical nucleotides in the primers and probes. However, although not obvious, one or several of these substitutions must account for the significantly lower sensitivity in the Q_NSAV assay compared with Q_nsP1. The Q_NSAV assay is thus not an optimal real-time PCR detection for Norwegian salmonid alphavirus under the conditions used herein, demonstrating that ideally more that one assay should be tested for a target template to ensure that the real-time PCR is sufficiently sensitive for its purpose.

The Q_nsP1 assay was 10–100-fold more sensitive than standard RT-PCR on all virus stocks. As a consequence of the increased sensitivity of the real-time PCR assays the number of positive field samples also increased. The Q_nsP1 and Q_NSAV assays were able to detect target RNA in six samples which were negative with standard RT-PCR. These six fish had high C_t-values and probably reflect either a carrier status of pancreas disease, pre-viraemia, or post-viraemia where the host is in the process of clearing the virus. The same pattern with increased sensitivity was observed with a real-time assay for detection of Infectious salmon anemia virus (ISAV) (Munir and Kibenge, 2004). They showed that all fish that were positive in the real-time assay, but negative in the corresponding conventional RT-PCR, had C_t-values 36 or higher, and proposed that the increased sensitivity in their real-time PCR assay would be useful for detecting subclinical ISAV infections. Preliminary data (personal observation) have shown that the Norwegian salmonid alphavirus resides in the infected fish in low, but stable numbers for at least 6.5 months after the initial infection, indicating that replication of Norwegian salmonid alphavirus occur in surviving fish after the viraemic phase. In view of the short production cycle period in seawater, salmon could thus be considered a life-long carrier of the virus. Interestingly, the lowest C_t-values from the Q_nsP1 assay on field samples (Table 2) are approximately the same as for the undiluted virus stocks. This indicates an extensive virus replication in the heart tissue of fish in a viraemic phase, although this cannot be verified without an adequate normalization of the above data.

The true amount of infectious virus particles is not necessarily detected by a real-time assay. It is likely that such an assay will over-estimate the number of infectious particles since viral RNA-target also is detected from replication intermediates, unpacked genomes, defective particles and free viral RNA from damaged cells. In the present study, the relationship between C_t-values from the real-time PCR assays and the $TCID_{50}$ from the viral stock dilution series was investigated. As expected, the viral RNA detected from dilutions corresponding to $TCID_{50}$ lower than 1.0. As little as 0.08 $TCID_{50}$ for salmon pancreas disease virus, 0.04 $TCID_{50}$ for sleeping disease virus and 0.01 for Norwegian salmonid alphavirus were detected, which suggests that a considerable amount of target RNA from the cell-cultures originate from non-infectious virus particles, or that the $TCID_{50}$ assays have low sensitivity.

From the plasmid dilution series it was demonstrated that the Q_nsP1 assay detects as few as 1-10 copies of linearized plasmid (ds DNA) containing the target sequence. Together with the observed slope values close to -3.34, this indicates that the PCR amplification in the assay is near optimal and is a very good candidate for use in absolute quantitation. The uncertainty of applying the Q_nsP1 assay in absolute quantitation of salmonid alphavirus target thus rests in the efficiency of the cDNA synthesis. As pointed out by Bustin and Nolan (2004) and Stahlberg et al. (2004), the efficiency of the reverse transcriptase step depends on the priming strategy, amount of RNA input, and choice of reverse transcriptase enzyme, which all varies among different genes. A validation of the Q_nsP1 assay for possible use in absolute quantification should first be clarified through serial dilutions of known numbers of salmonid alphavirus RNA-templates, with subsequent cDNA synthesis and real-time PCR. In most cases, however, the need for absolute quantification of the virus is not required, and it is sufficient to merely document the relative changes of salmonid alphavirus templates between varying experimental conditions (Bustin, 2000; Mackay et al., 2002; Pfaffl, 2001). This can be achieved by monitoring simultaneously a non-regulated reference target; either internal or added externally.

A real-time PCR technique for routine detection of all subtypes of salmonid alphavirus has been developed (Q_nsP1-assay), and has further potential use a quantitative tool in field or experimental studies of salmonid alphaviruses. The method is rapid, sensitive and specific, without amplifying product from other piscine viruses occurring in the salmon industry. Further optimization of the real-time assays could result in a reliable multiplex assay to specifically detect any of the three subtypes of salmonid alphavirus in the same reaction. The most significant advantages of real-time over conventional diagnostics of pancreas disease are time and labour savings. Where the traditional diagnosis of pancreas disease usually takes minimum one (standard RT-PCR) or several days (histopathology and/or cell culturing), the real-time PCR assay reported here can provide a reliable confirmation of salmonid alphavirus within 5 h. Additional time-savings can be achieved by using a commercial available one-step real-time RT-PCR, and thereby exclude the need for a separate cDNA synthesis step. The Norwegian salmonid alphavirus appears to be the only salmonid alphavirus subtype occurring in Norway, and is at present not reported elsewhere (present study; Hodneland et al., 2005). The salmon pancreas disease virus subtype is restricted to the UK, whereas the sleeping disease virus is reported from both France and UK (Branson, 2002). By using the above real-time PCR asssays it is now possible to easily distinguish between the subtypes of salmonid alphavirus, which is important for possible prevention of introduction of a specific subtype into new countries or areas where this subtype has not been reported previously. Also, in countries where a specific salmonid alphavirus is naturally occurring, early detection and confirmation of a suspected salmonid alphavirus aetiology of diseased salmonids is important for in-farm man-

agement, and may provide valuable information to slow down or stop the spread of the disease to new locations or regions.

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