A capsid gene-based real-time reverse transcription polymerase chain reaction assay for the detection of marine vesiviruses in the *Caliciviridae*

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ABSTRACT

A real-time reverse transcription polymerase chain reaction (rtRT-PCR) assay was developed for the identification of marine vesiviruses. The primers were designed to target a 176-nucleotide fragment within a highly conserved region of the San Miguel sea lion viruses (SMSVs) capsid gene. The assay detected viral RNA from nine marine vesivirus serotypes described previously, including two serotypes (SMSV-8 and -12) not identified with presently available molecular assays, a highly related bovine vesivirus strain (Bos-1), a mink vesivirus strain (MCV), and two novel genotypes isolated recently from Steller sea lions (SSL V810 and V1415). The real-time assay did not amplify sequences from the corresponding genomic regions of feline calicivirus (also in the genus *Vesivirus*) and representative members of the genus *Norovirus*. The rtRT-PCR assay described below may prove useful as a diagnostic tool for the detection of currently circulating, emerging and previously described marine vesiviruses in clinical samples, especially when large numbers are screened in surveillance studies of these restricted viruses.

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1. Introduction

Viruses within the genus *Vesivirus* of the family *Caliciviridae* are found in many species of marine and terrestrial vertebrates. The genus *Vesivirus* is divided presently into two major virus species: *Vesicular exanthema of swine virus* (VESV) and *Feline calicivirus* (FCV). The VESV species was identified following the occurrence of outbreaks of vesicular disease in swine in California in 1932 that showed striking similarity to foot-and-mouth disease (Traum, 1936). Thousands of pigs were destroyed before foot-and-mouth disease virus (FMDV) was excluded as the etiologic agent, and a new virus, designated VESV was associated with the disease. It was later proposed that VESV was introduced into pig herds by the accidental contamination of feed with marine mammal and fish products harboring serologically related vesiviruses (Smith et al., 1973; Smith and Boyt, 1990).

Vesiviruses isolated from marine mammals are known commonly as San Miguel sea lion viruses (SMSVs) and marine mammals are considered natural hosts (Smith et al., 1973). Vesicular disease and reproductive failure are the most common signs when illness occurs. Species of marine mammals found infected with vesiviruses include sea lions, seals, walrus, whales, and dolphins (Smith and Boyt, 1990). Fish may also be natural hosts for the vesiviruses. Several serotypes of SMSV, including SMSV-7 which caused classical vesicular exanthema disease in exposed swine have been isolated from the Opaleye fish (*Girella nigricans*) (Smith et al., 1980b). The Opaleye fish has also been shown to support the replication of SMSV-5 when challenged under experimental conditions (Smith et al., 1980a). Other strains of vesiviruses closely related to the marine vesiviruses have been identified in terrestrial animals. These include isolates from rabbits (Martin-Alonso et al., 2005), skunks (Seal et al., 1995b), reptiles and amphibians (Smith et al., 1986), monkeys (Smith et al., 1978, 1985), and humans (Smith et al., 1998, 2006, 1978). More recently, evidence was reported that vesivirus infection may be associated with abortion in horses (Kurth et al., 2006). To date, more than 40 different serotypes and untyped marine vesiviruses that infect both marine and terrestrial animals have been described (Smith and Boyt, 1990), but serotyping reagents are not widely available for characterizing new viruses.

Of economical and regulatory significance is the resemblance of vesivirus disease in swine to FMD, classified as a Foreign Animal Disease (FAD) in U.S. livestock and caused by FMDV, a picornavirus. For these reasons, the marine vesiviruses in the VESV species are considered restricted in the United States and have been designated as Foreign Animal Disease Agents (Bankowski, 1965; Smith and Boyt,

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1990). It is crucial therefore, to have rapid, specific and sensitive diagnostic assays to detect and identify vesiviruses associated with blisters or vesicles in order to distinguish them from those caused by FMDV. Current diagnostic assays for the identification of marine vesiviruses include: (1) conventional virological techniques, such as virus isolation in cell cultures and electron microscopy (Smith et al., 1973, 1974), (2) serological methods, including virus neutralization (Smith and Akers, 1976; Smith and Boyt, 1990) and enzyme linked immunosorbent assays (Ferris and Oxtoby, 1994), and (3) molecular assays, such as conventional reverse-transcription polymerase chain reaction (RT-PCR) (Reid et al., 1999) and more recently, a real-time RT-PCR (rtRT-PCR) (Reid et al., 2007). Although the latter assay targeted sequences corresponding to the highly conserved RNA dependent RNA polymerase (RdRp) gene, it failed to detect and identify marine vesiviruses of two serotypes.

The purpose of the present study was to develop an improved rtRT-PCR assay that could detect a broad range of marine vesiviruses. Available marine vesivirus sequences were aligned, and a primer pair and specific probe were selected from a highly conserved region of the capsid gene. The detection efficiency and specificity of the rtRT-PCR was examined against a panel of well-characterized vesiviruses and noroviruses, and the new assay performed well in the specific detection of marine vesiviruses.

2. Materials and methods

2.1. Viruses and RNA

The marine vesiviruses used to develop the real-time (rt) RT-PCR assay were propagated in African green monkey kidney (Vero) cell cultures and total RNA was extracted at the height of cytopathogenic changes using TRIzol-LS (Invitrogen). Briefly, the infected monolayers were lysed in TRIzol-LS and phase separated by chloroform treatment. Total RNA was precipitated with isopropyl alcohol and the resulting RNA pellet was dissolved in RNase-free water. The viruses used in the validation of the real-time assay were 10 marine vesiviruses described previously including SMSV-1, -2, -4, -5, -6, -8, -12,-13, -14, bovine calicivirus Bos-1 (Smith et al., 1983; Smith and Boyt, 1990), and two recently characterized marine vesiviruses isolated from Steller sea lions (SSL) from Alaska; SSL V810 and SSL V1415 (McClenahan et al., 2008). Several other members of the genus *Vesivirus* were assayed also to determine the specificity of the rtRT-PCR assay, including four isolates of *Feline calicivirus* (FCV) propagated in Crandall-Reese feline kidney (CRFK) cell cultures. Three FCV isolates were recovered from cats in Florida (C01, C46, and C58) (Weeks et al., 2001), and the Urbana FCV isolate was described previously (Sosnovtsev and Green, 1995). A vesivirus isolated from mink, mink calicivirus (strain MCV9) (Evermann et al., 1983; Guo et al., 2001), was included in the analysis. RNA samples extracted from viruses representing three genogroups (G) of the genus *Norovirus* within the *Caliciviridae* were tested and included human noroviruses Norwalk virus (G1.1) (Kapikian et al., 1972), DC-56 (GII.1) (Brandt et al., 1983), and MD104-4 (GII.4) (Green et al., 2002), and murine norovirus strains NIH-MNV 2409 (Perdue et al., 2007) and MNV-1 (Karst et al., 2003) in Genogroup V. Vesicular stomatitis virus (VSV) serotype Indiana was used as a control to rule out nonspecific detection of an unrelated virus that produces clinical signs in swine similar to those induced by marine vesiviruses. The presence of VSV RNA was verified by the RT-PCR method of Rodriguez et al. (1993).

2.2. Standards for rtRT-PCR assay

Two standards were developed for evaluation of the rtRT-PCR assay. First, a 176-bp capsid gene fragment from SSL vesivirus strains V810 or V1415 (corresponding to nucleotides 5677–5853 and 5671–5847, respectively) was PCR amplified, and cloned into the plasmid vector pGEM T Easy (Promega) under the transcriptional control of the T7 promoter. The plasmids were purified, quantified by spectrophotometry (Pharmacia Biotech) and a series of 10-fold dilutions were prepared. The plasmid copy number at each dilution was calculated as previously described (Wilhelm and Truyen, 2006) using standard methods (Applied Biosystems, 2003; Bookout et al., 2006), and the dilution series was analyzed in the rtRT-PCR assay to generate a standard curve. Second, total RNA was extracted from cells infected with SSL vesivirus isolates V810 and V1415 as described above, and further purified using the RNeasy Mini Kit (Qiagen). The RNA was quantified using a NanoDrop Spectrophotomer (Labtech, Dublin, Ireland), and 10-fold serial dilutions were prepared in water. This RNA standard was used in optimization studies and in experiments as an RNA template control.

2.3. Reverse transcription

Total RNA (500–1000 ng) extracted from vesivirus-infected cells was quantified by spectrophotometry and reverse transcribed (RT) into complementary DNA (cDNA). The RT reaction volume was 20 μl and contained 0.5 μl of random hexanucleotide primers (Invitrogen), 40 U of ribonuclease inhibitor RNase OUT (Invitrogen), and 1 μl of Superscript II (Invitrogen). The cDNAs were stored at −80 °C.

2.4. Primers and probe design

A multiple alignment of the capsid gene sequences of several vesiviruses available in the GenBank database of the National Center for Biotechnology Information (NCBI) was performed with the MegAlign function of the Lasergene software (DNASTAR) to identify conserved sequences (Fig. 1). These viruses included SMSV-1 (U15301), SMSV-4 (M87482), SMSV-17 (U52005), VESV (U76874), primate calicivirus (Pan-1) (AF091736), walrus calicivirus (WCV) (AF321298), and rabbit vesivirus (RaCV) (AJ866991). Also included were vesivirus isolates SSL V810 (EF193004) and SSL V1415 (EF195384) (McClenahan et al., 2008). Conserved sequences were identified and used to design consensus primers, and a 16-nucleotide (nt) fluorogenic probe. Primers were as follows: FP CR792 5′-ATGGCTACTACTCAIACGCT-3′ (beginning at nt 5671 in VESV-A48) and RP CR793 5′-CAGTTGAAIGGATCATCACA-3′ (beginning at nt 5831 in VESV-A48). The melting temperatures of the two primers were 55 °C and 52 °C, respectively. This primer pair was used also in a standard RT-PCR assay to amplify the capsid region for direct sequence analysis in the verification of selected strains, and for evaluation of the efficiency of standard RT-PCR versus that of the new real-time RT-PCR assay. The fluorogenic probe was designed with a FAM dye label at the 5′-end and an Iowa Black quencher at the 3′-end and also incorporated locked nucleic acids (LNA) (Integrated DNA Technologies) to increase the melting temperature of the probe in the assay and improve specificity and stability (Ugozzoli et al., 2004). The fluorogenic probe was: 6-FAM/A+CCT+CGAA+TTT+CT+CTT/IABlkFQ (beginning at nt 5705 in VESV-A48), with the added LNA shown as + signs. The melting temperature of the probe was 59 °C and the expected size of the amplified rtRT-PCR product was 176-bp in length.

2.5. Real-time RT-PCR assay

The rtRT-PCR assay was developed initially in a two-step platform at the Department of Infectious Diseases and Pathology, University of Florida and performed using the SmartCycler II (Cepheid), a portable thermocycler with a 16-sample capacity. The
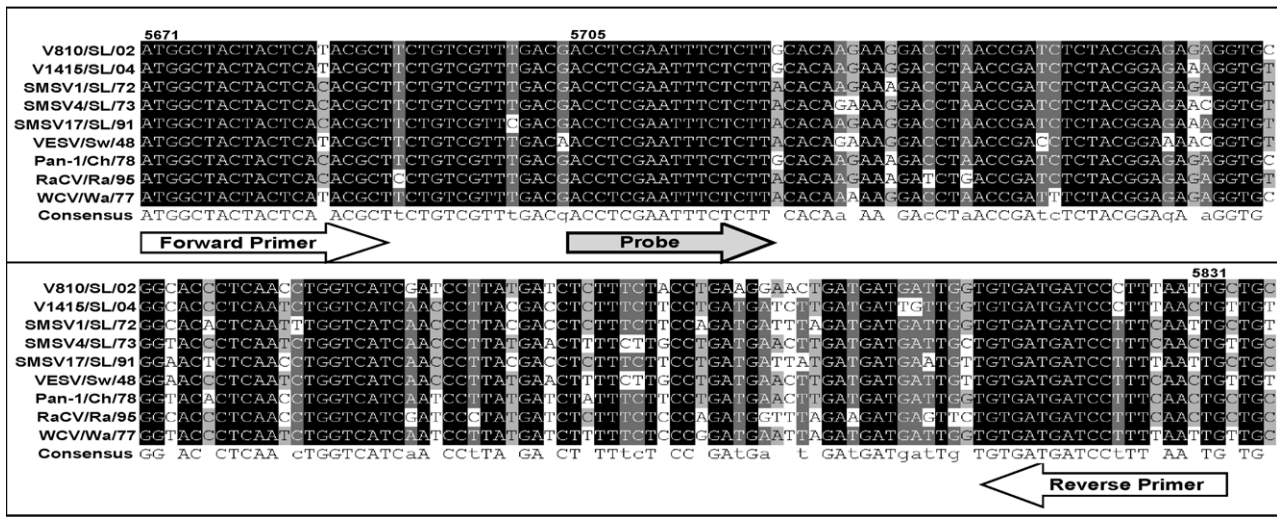
Fig. 1. Multiple alignment of the highly conserved region A of the capsid protein gene of marine vesiviruses used to design primers and probe for the real-time RT-PCR assay. The location of the forward and reverse primers and the fluorogenic probe is indicated. The genomic location is indicated according to the sequence of Sw/VeV/VESVA48/1948/US (NCBI Reference Sequence NC_002551). Identifiers (strain name/host/year of isolation) are as follows: SL, sea lion; Sw, swine; Ch, Pygmy chimpanzee; Ra, European rabbit; Wa, Walrus.

assay was evaluated also in a one-step platform used routinely in the Laboratory of Infectious Diseases at NIH that utilized the 96-sample capacity 7900HT Fast Real-time PCR machine (Applied Biosystems [ABI]).

The RNA tested in the two-step platform was first reverse-transcribed in a separate reaction, as described in Section 2.3, and then added to the real-time PCR mixture. The mixture (performed in a final volume of 25 μl) contained 3.5 mM MgCl$_2$, 200 nM of each forward and reverse primer, 240 nM of the fluorogenic probe, 1 mM of each dNTP, 1 U of Taq DNA polymerase (New England Biolabs), and 2 μl of cDNA. The cycling program consisted of an initial denaturation at 95 °C for 120 s followed by 40 cycles of 2 s melting at 95 °C, 30 s of annealing at 47 °C, and 10 s extension at 72 °C. The fluorescent signal from the probe was measured during the elongation step of each cycle.

The one-step platform used a commercial Brilliant II QPCR Mastermix with Rox (Stratagene). The reaction mixture (performed in a final 25 μl volume) contained 2.5 mM MgCl$_2$, 400 nM of each forward and reverse primer, 400 nM of the fluorogenic probe, 0.8 mM of each dNTP, 1.25 U of SureStart Taq polymerase, 1 μl StrataScript reverse transcriptase, 500 nM ROX reference dye, and 5 μl of RNA template. The cycling program consisted of an initial incubation at 45 °C for 30 min, followed by incubation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 48 °C for 1 min, and 72 °C for 30 s. The fluorescent signal from the probe was measured throughout the PCR assay.

2.6. Optimization of the real-time reaction and specificity

The optimal concentrations of Mg^{2+} ions, primers and fluorogenic probe were initially evaluated in the two-step rtRT-PCR assay using the SmartCycler II machine. The optimal concentration of each reagent was considered to be the quantity that produced the lowest cycle threshold (Ct) value using as positive control 1 μg of total RNA obtained from Vero cell cultures infected with SSL vesiviruses V810 and V1415. Two independent rtRT-PCR runs were used to evaluate each variable, and within each run, the variables were tested in duplicate. The four Ct values for each variable were averaged to obtain one final Ct value for each concentration tested. For the one-step rtRT-PCR assay using the ABI machine, conditions optimized for norovirus detection were employed as described in Section 2.5. The specificity of the rtRT-PCR for marine vesiviruses

was determined by the analysis of RNA derived from the viruses described in Section 2.1.

2.7. Limit of detection versus infectivity

The limit of detection (LOD) of the viral RNA as measured by rtRT-PCR was compared to the number of infectious units of the virus measured in cell culture. The SSL marine vesiviruses V810 and V1415 were propagated in Vero cell cultures using undiluted supernatants from the second passage, and harvested 24 h post-infection. The cells and supernatants were frozen and thawed twice, and clarified by centrifugation at 12,000 × g for 10 min at 4 °C. The supernatants containing infectious vesiviruses (virus stocks) were diluted serially 10-fold in Dulbecco's minimal essential medium (DMEM) and analyzed in virus titration assays. The RNA from the original virus stock was extracted with TRIzol-LS, as described above, diluted serially 10-fold, and tested in PCR-based diagnostic assays.

Two methods were used for viral titration: an end-point dilution method to determine tissue culture infectious doses 50 (TCID$_{50}$), and a plaque assay to determine plaque forming units (PFU) per ml. In the end-point dilution method, 100 μl of each 10-fold serial dilution were added to eight separate wells in a 96-well cell culture plate. Then, 20,000 Vero cells in 100 μl of DMEM supplemented with 1% fetal bovine serum (FBS) were added to each well and the plates were incubated at 37 °C in an atmosphere of 5% CO$_2$ and evaluated for cytopathogenic changes after 3 and 7 d. In the plaque assay, Vero cells were plated in 6-well plates and incubated at 37 °C in an atmosphere of 5% CO$_2$ until confluent. One milliliter of each viral dilution was then added directly onto the cell monolayers, in duplicate, and viral adsorption was allowed for 1 h at 37 °C. The inocula were removed and replaced with a 1% agarose overlay in DMEM supplemented with 1% FBS. The plates were incubated at 37 °C in 5% CO$_2$ for 2 d to allow plaques to form, and the monolayers were fixed overnight with 10% neutral buffered formalin. The agarose overlays were removed and the cells stained with a solution of 0.2% crystal violet. Plaques were counted, averaged between the duplicate wells, and the titer was expressed in PFU/ml. The RNA extracted from the virus stock was diluted as described above and tested in the one-step rtRT-PCR assay. The last dilution with a positive signal in the PCR assay or infectivity assay was considered the end-point titer, and expressed as the reciprocal of the dilution.

3. Results

3.1. Reaction conditions of the real-time RT-PCR assay

The vesivirus primers and fluorogenic probe targeted against the capsid gene were screened initially for the detection of V810 and V1415 RNA by rtRT-PCR in a two-step reaction format, in which the cDNA was synthesized in a separate RT reaction prior to the PCR step. Following the demonstration of a positive signal, the assay was optimized further (data not shown). The optimal concentrations and annealing temperatures of the primers in the two-step assay were established as 200 nM per reaction and 47 °C, respectively, while the optimal probe concentration was determined to be 240 nM per reaction. The optimal Mg^{2+} concentration for the PCR component of the two-step assay was determined to be 3.5–4 mM per reaction.

We next evaluated the performance of the vesivirus primers and fluorogenic probe in a one-step format, in which the RT and PCR reactions were conducted in a single tube. The initial screening assay with V810 and V1415 RNA showed that a one-step rtRT-PCR reaction employing conditions that had been optimized previously with norovirus primers and template performed well (data not shown). The vesivirus RNA templates prepared from virus-infected cells could be detected efficiently beginning at a 10^{-3} dilution (0.5–1 ng RNA). The concentrations of vesivirus primers and temperature used in the one-step assay were 400 nM per reaction and 48 °C, respectively. The probe was used at a concentration of 400 mM, and the concentration of Mg^{2+} was 2.5 mM per reaction. This finding simplified the concurrent analysis of vesivirus and norovirus samples in the same 96-well plate.

3.2. Specificity

The specificity of the rtRT-PCR assay was evaluated using two panels of calicivirus RNA available in different laboratories (Table 1). At the University of Florida (Panel 1), marine vesiviruses SMSV-1, -2, -4, -5, -6, -8, -12, -13, -14, bovine calicivirus Bos-1, isolates SSL V810 and V1415, and three isolates of FCV were analyzed in the two-step rtRT-PCR assay (Table 1). All 12 marine vesivirus strains were positive in this assay with Ct values ranging from 12 to 31. The three FCV strains (representing a separate species in the genus *Vesivirus*) were not detected in the assay. In addition, reactivity was not observed with RNA derived from vesicular stomatitis virus, an unrelated virus associated also with vesicular lesions. At the NIH (Panel 2), vesivirus strains SSL V810, V1415, FCV, and MCV were analyzed in the one-step rtRT-PCR assay (Table 1). The Ct values of SSL V810 and V1415 were 17 and 16, respectively, when the RNA was analyzed at a 10^{-3} dilution. The assay detected mink calicivirus (Ct value, 30), presently classified as an unassigned species in the genus *Vesivirus*, and again, did not detect FCV. Human norovirus strains GI.1, GII.1, and GII.4 and two distinct strains of murine norovirus (GV) were not detected in the one-step vesivirus rtRT-PCR.

3.3. Viral infectivity, limit of detection, and standards

The ability to measure virus infectivity in cell culture provided an opportunity to compare the quantification of viral RNA by rtRT-PCR with the quantification of infectious units of the virus stock from which it was derived. Virus stocks were prepared from SSL strains V810 and V1415, and the titers determined by end-point dilution in microtiter tissue culture plates to calculate the $TCID_{50}$ and by plaque assay on a cell monolayer to quantify the number of PFU per ml (Table 2). The V810 virus titer was determined to be $10^{9.75}$ $TCID_{50}$ and 10^{9} PFU/ml and the V1415 virus titer was $10^{8.5}$ $TCID_{50}$ and 6×10^{8} PFU/ml. The RNA was extracted from the

Table 1
Real-time RT-PCR analysis of two RNA panels that include members of the marine vesivirus group.

Virus tested	Ct value[a]
University of Florida RNA panel (2-step, Smart Cycler II machine)	
SMSV-1	12
SMSV-2	15
SMSV-4	12
SMSV-5	17
SMSV-6	14
SMSV-8	31
SMSV-12	12
SMSV-13	15
SMSV-14	18
Bovine calicivirus, Bos-1	12
SSL V810	16
SSL V1415	13
FCV-C01	0
FCV-C46	0
FCV-C58	0
VSV (Indiana) control	0
NIH RNA panel (1-step, ABI 7900 machine)	
SSL V810 (10^{-3} dilution RNA)	17
SSL V1415 (10^{-3} dilution RNA)	16
MCV-9	30
FCV-Urbana	0
Murine norovirus-NIH 2409	0
Murine norovirus- MNV-1	0
Norovirus GI.1-Norwalk	0
Norovirus GII.1-DC56	0
Norovirus GII.4-MD104-4	0

Abbreviations: SMSV, San Miguel sea lion virus; SSL, Stellar sea lion; FCV, feline calicivirus; MCV, mink calicivirus.

[a] The 2-step and 1-step assays were performed as described in Section 2. A Ct cut-off value of 35 or less was considered positive in the rtRT-PCR.

virus stock used in the infectivity titration and diluted, and the presence or absence of RNA at each dilution was analyzed by rtRT-PCR and standard RT-PCR to determine the limit of detection (LOD). The rtRT-PCR was positive for the detection of RNA at similar dilutions in which infectious virus was detected in cell culture (Table 2), although the $TCID_{50}$ assay appeared to be the most sensitive of the assays examined. The same RNA samples were analyzed in a standard RT-PCR assay and the products visualized in an agarose gel (data not shown). The highest dilution at which RNA was detected was the 10^{-4} dilution for both viral isolates (Table 2), indicating a lower sensitivity of the standard RT-PCR compared with the rtRT-PCR assay.

The recombinant plasmid encoding the 176-nt capsid region of V810 was used to generate a standard curve in the one-tube rtRT-PCR assay that ranged from 1×10^{2} to 1×10^{8} plasmid copies/reaction (Fig. 2A). An R^2 value of 0.99 was obtained (Fig. 2B). The slope of the amplification plots of the serially diluted plasmid DNA was used to calculate the efficiency of the reaction from the equation $-1 + 10^{(-1/\text{slope})}$ (Applied Biosystems, 2003; Bookout et al., 2006), and the efficiency was 97.24%. The RNA copy number at the LOD was calculated to be 264 RNA copies/reaction for V810 (Table 2). A similar analysis of the V1415 RNA with the V1415 capsid region plasmid as standard (data not shown) yielded a calculated 2000 RNA copies/reaction for V1415 at the LOD.

4. Discussion

This report describes a new rtRT-PCR assay for the detection of marine vesiviruses. The assay amplified and identified twelve distinct "marine" vesiviruses, including two recently described vesiviruses from Steller sea lions (McClenahan et al., 2008), and two vesiviruses not previously detected with other molecular assays (Reid et al., 1999, 2007). The assay detected a mink calicivirus strain

Table 2
Comparison of virus infectivity assays, real-time RT-PCR, and standard RT-PCR, in the limit of detection (LOD) of marine vesivirus strains SSL V810 and SSL 1415.

| Assay	Sample analyzed	Parameter measured	V810[a] end-point titer	V1415[a] end-point titer
$TCID_{50}$	Virus stock	Infectivity	$1 \times 10^{9.75}$	$1 \times 10^{8.5}$
PFU/ml	Virus stock	Infectivity	1×10^9	6×10^8
Real-time PCR	RNA	Detectable RNA	10^9 (264 copies RNA)	10^8 (2000 copies RNA)
Standard PCR	RNA	Detectable RNA	10^4	10^4

[a] End-point titer expressed as reciprocal of highest dilution with positive signal in indicated assay

tentatively assigned to the genus *Vesivirus*. The rtRT-PCR did not amplify four different isolates of the nonmarine vesivirus species FCV and five viruses belonging to the genus *Norovirus* within the *Caliciviridae*.

The real-time assay targeted the highly conserved A region at the 5′-end of the capsid precursor protein gene contained in ORF 2 (Neill, 1992) and although not tested with all previously described marine vesiviruses, should detect the known and serologically untyped marine vesiviruses with complete capsid gene sequences available in the NCBI database. The fluorogenic probe designed for use in the assay hybridized efficiently to the well-conserved region of 16 nucleotides in region A of ORF 2. Furthermore, the incorporation of LNAs into the fluorogenic probe, which has been reported to increase the specificity and stability of such probes (Ugozzoli et al., 2004), most likely contributed to the improved performance of the new rtRT-PCR assay.

The rtRT-PCR conditions were optimized initially in the two-step assay. Varying concentrations of the fluorogenic probe (20–240 nM)

did not appreciably alter the Ct values in the assay, and within this range, we chose a probe concentration of 240 nM, in line with that used in a previously published real-time assay (Reid et al., 2007), and to allow for the increased detection of very low amounts of target viral RNA. Primer concentration seemed to cause more variation in the Ct values; with low concentrations such as 50 nM increasing Ct values and high concentrations of the order of 400 nM to 1 μM causing false positive reactivity even in the absence of cDNA template (data not shown). An intermediate concentration of 200 nM of each primer was chosen, similar to that of a recently published rtRT-PCR assay (Reid et al., 2007). The primers and probe in this study performed efficiently in a one-step assay that had been optimized for the detection of human noroviruses, even though the primer and probe concentrations were higher (400 nM). These data suggest that optimal conditions may vary among laboratories according to the reagents used and the type of thermocycler available, and further optimization should be considered if Ct values are higher than expected with control RNA.
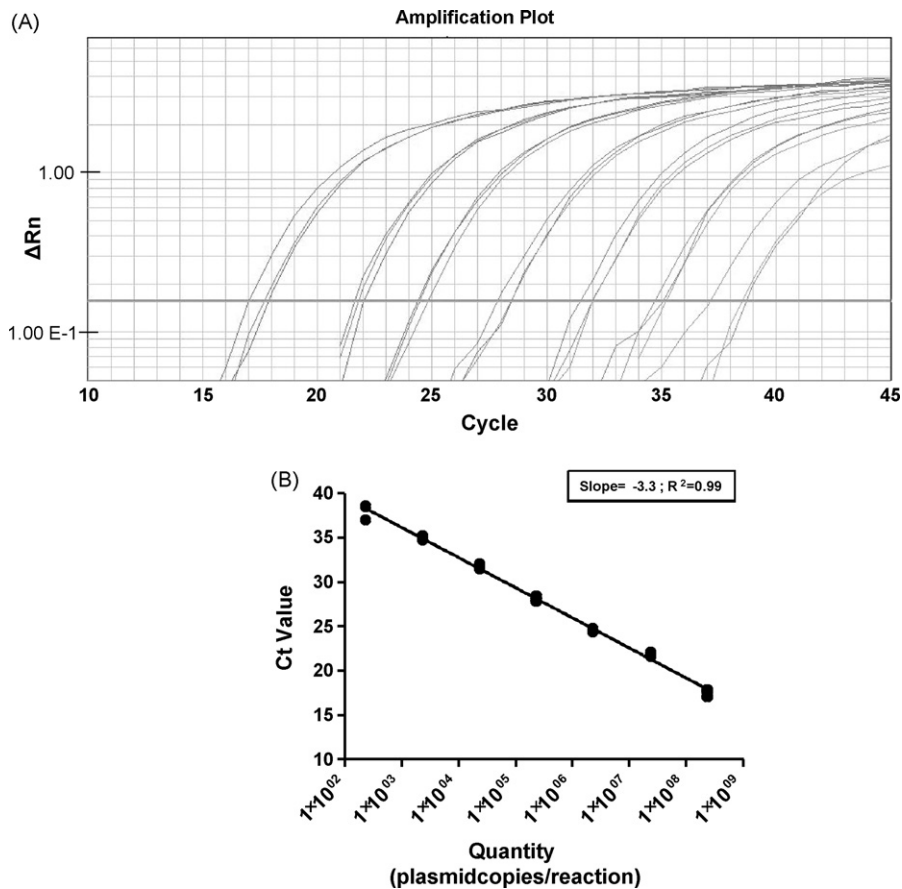
Fig. 2. Dynamic range of the real-time RT-PCR assay (one-step platform) with recombinant plasmid DNA for the detection of a 176-bp target fragment of the capsid gene of the V810 isolate of SSL marine vesivirus. (A). Serial 10-fold dilutions of this plasmid containing the cloned target fragment (ranging from 10^8 to 10^2 copies per reaction mixture) were tested in triplicate. The copy numbers of the target sequences are indicated with the corresponding Ct values ranging from 17.30 ± 0.34 to 44.56 ± 0.45. (B) The coefficient of determination (r^2) was 0.99 as shown in the standard curve plot.

Quantitation by the rtRT-PCR assay was accomplished by generating a standard curve with a recombinant plasmid carrying the 176-bp capsid gene target fragment. The presence of subgenomic RNA in the total RNA extracted from virus-infected cells made precise calculation of full-length genome equivalents difficult, so an alternative approach was used to calculate the limit of detection (LOD). The rtRT-PCR assay detected the V810 viral RNA target diluted up to 10^{-9}, which corresponded to 264 copies of RNA as calculated by comparison of the quantity of total RNA with the plasmid standard curve. The lower-titered V1415 displayed a lower LOD, as expected. The LODs observed in this study are consistent with those obtained in rtRT-PCR assays for the detection of other caliciviruses (Reid et al., 2007; Wilhelm and Truyen, 2006), and should allow for the quantitation of viral RNA in tissue samples containing low amounts of virus.

An rtRT-PCR assay previously described for the detection of marine vesiviruses (Reid et al., 2007) in which the RNA dependent RNA polymerase gene was targeted, failed to amplify SMSV-8 and -12. These serotypes are considered quite distinct from most marine vesivirus serotypes, as specific antisera against these viruses do not cross-react with other SMSV serotypes in immunoassays (Seal et al., 1995a). A set of primers designed for a conventional RT-PCR for marine vesiviruses also failed to amplify SMSV-8 and -12 (Reid et al., 1999). As mentioned above, both SMSV-8 and -12 were amplified and correctly identified as marine vesiviruses in the rtRT-PCR assay described here. The region of the capsid gene targeted corresponded to the highly conserved region A of the capsid gene (Neill, 1992), and most likely represents a better target region than the RNA polymerase region targeted in other assays. However, because of the quasispecies evolution of the capsid gene of vesiviruses (Radford et al., 1998), it may be necessary in the future to refine the new rtRT-PCR assay to correct for minor changes in the genome of novel and emerging marine vesiviruses.

In summary, a new rtRT-PCR assay was developed for the detection of marine vesiviruses that performed well in two formats. When more than one RNA virus is suspected, i.e., enteroviruses, coronaviruses, paramyxoviruses, etc., the two-step assay may be preferred in a diagnostic laboratory as the generated cDNA may then be used in several different PCR assays. Conversely, when the primary goal is the detection of marine vesiviruses in clinical or research specimens, the one-tube assay may be preferred. The assay amplified successfully a 176 nucleotide fragment from a highly conserved region of the capsid gene after reverse transcription of total RNA derived from cell cultures infected with 12 different marine vesiviruses. The assay did not amplify closely related terrestrial vesiviruses such as FCV and five members of the genus *Norovirus*. The new rtRT-PCR assay is at least 100,000 times more sensitive than a conventional RT-PCR assay utilizing the same primers. Although not evaluated in the present report, this novel diagnostic assay has the potential for use as a rapid, sensitive, and specific assay to detect known and novel marine vesiviruses in field clinical samples such as oropharyngeal and rectal swabs and blister fluids.

Acknowledgements

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