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Real-time RT-PCR for detection, identification and absolute quantification of viral haemorrhagic septicaemia virus using different types of standards

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ABSTRACT: In the present study, 2 systems of real-time RT-PCR — one based on SYBR Green and the other on TaqMan — were designed to detect strains from any genotype of viral haemorrhagic septicaemia virus (VHSV), with high sensitivity and repeatability/reproducibility. In addition, the method was optimized for quantitative purposes (qRT-PCR), and standard curves with different types of reference templates were constructed and compared. Specificity was tested against 26 isolates from 4 genotypes. The sensitivity of the procedures was first tested against cell culture isolation, obtaining a limit of detection (LD) of 10^0 $TCID_{50}$ ml^{-1} (100-fold below the LD using cell culture), at a threshold cycle value (Ct) of 36. Sensitivity was also evaluated using RNA from crude (LD = 1 fg; 160 genome copies) and purified virus (100 ag; 16 copies), plasmid DNA (2 copies) and RNA transcript (15 copies). No differences between both chemistries were observed in sensitivity and dynamic range. To evaluate repeatability and reproducibility, all experiments were performed in triplicate and on 3 different days, by workers with different levels of experience, obtaining Ct values with coefficients of variation always <5. This fact, together with the high efficiency and R^2 values of the standard curves, encouraged us to analyse the reliability of the method for viral quantification. The results not only demonstrated that the procedure can be used for detection, identification and quantification of this virus, but also demonstrated a clear correlation between the regression lines obtained with different standards, which will help scientists to compare sensitivity results between different studies.

KEY WORDS: qPCR · Viral haemorrhagic septicaemia virus · Quantification

INTRODUCTION

Viral haemorrhagic septicaemia virus (VHSV) belongs to the family *Rhabdoviridae*, genus *Novirhabdovirus*, and has been associated with significant losses of wild and cultured freshwater and marine fish species. Since the first isolation of VHSV in Denmark (Jensen 1965), the virus has been detected in many countries worldwide, and has been isolated from over 70 fish species (OIE 2012).

The genome is composed of a single-stranded, non-segmented negative sense RNA genome of 11 to 12 kb (Schütze et al. 1999). It contains 6 open reading frames (3′-N-P-M-G-NV-L-5′), corresponding to the nucleocapsid- (N), phospho- (P), matrix- (M), glyco- (G), non-virion (NV) and polymerase (L) proteins. Upon infection of the host, the 6 genes are transcribed progressively from the 3′ to 5′ end of the template viral RNA, and in decreasing molar frequencies (Kurath & Leong 1985). Therefore, due to the initial

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position of the N-gene in the VHSV genome, N transcripts are the most abundant during viral infection and therefore have been the target of several molecular assays used to detect VHSV (López-Vázquez et al. 2006, Matejusova et al. 2008, Cutrín et al. 2009, Hope et al. 2010).

Due to the high mortalities and significant losses that VHSV causes in aquaculture facilities, rapid and reliable methods for VHSV detection are needed to prevent the spread of the disease. The currently used and accepted international standards for detection of VHSV (OIE 2012), including an initial procedure of viral isolation in cell culture, followed by reverse transcriptase polymerase chain reaction (RT-PCR), and nucleotide sequencing to confirm the presence of VHSV as the infectious agent. In recent years, real-time quantitative PCR (qPCR) has emerged as a powerful technique for the diagnosis of viral diseases, providing a tool to detect and quantify virus. In addition, qPCR is a reproducible and highly specific assay for the detection and quantification of the virus and it is also less time-consuming than any other method (Dopazo & Bandín 2011). In this regard, qRT-PCR is a commonly used method for the detection of many RNA fish viruses, including VHSV and other fish rhabdoviruses, such as spring viraemia of carp virus (SVCV) and infectious haematopoietic necrosis virus (IHNV) (Purcell et al. 2006, Yue et al. 2008).

For the detection of PCR products during qRT-PCR, 2 chemistries are most commonly used: the DNA binding fluorophore SYBR Green I (Workenhe et al. 2008, Cutrín et al. 2009, Jiang et al. 2014) and the sequence-specific fluorescently labelled TaqMan probes (Chico et al. 2006, Matejusova et al. 2008, Garver et al. 2011).

Due to the limitations of previously published methods in terms of their specificity, sensitivity or applicability to the detection of all known genotypes of VHSV, we aimed to develop a universal assay for detection, identification and quantification of this virus in cell cultures. The procedure was designed for both systems, based on amplification of a 163 bp fragment of the nucleoprotein (N) gene, to detect any genotype of VHSV and to improve the reliability of the diagnosis. The methods were evaluated in terms of sensitivity, specificity and repeatability and reproducibility. In addition, this report describes the comparison of plasmid DNA, *in vitro* transcribed RNA, purified virus and crude virus as standards for absolute viral quantification.

MATERIALS AND METHODS

Virus and cells

A total of 35 VHSV strains from genotypes I (including sublineages Ia, Ib, Id and Ie), II, III and IV (including sublineages IVa, IVb and IVc), including reference strains and local isolates, were assayed *in silico* and/or *ex vivo* (Table 1). In addition, 2 strains of IHNV type 1 (River Butte 91 and Applegate River 93) kindly provided by Dr. J. A. Leong (Hawaii Institute of Marine Biology, Kaneohe, Hawaii, USA) were included as negative controls. For viral propagation, epithelioma papulosum cyprini (EPC) cells, purchased from the European Collection of Cell Cultures (ECACC 93120820, Salisbury, UK), were employed. The cells were cultured at 25°C in minimum essential medium (EMEM) supplemented with 10% foetal bovine serum (FBS), penicillin and streptomycin (100 IU ml^{-1} and 100 µg ml^{-1}, respectively). When the monolayers showed around 90% confluence, the medium was substituted by EMEM without FBS, and the virus was inoculated at a multiplicity of infection (MOI) of 0.1 to 1; infected monolayers were incubated at 15°C until the development of cytopathic effect. Viral titration was performed as described previously (Dopazo et al. 2002).

RNA extraction

The extraction of RNA was performed using Trizol® LS reagent (Gibco, BRL) as described by the manufacturer. The extracted RNA was finally resuspended in 50 µl of diethyl pyrocarbonate (DEPC)-treated water, and quantified by absorbance measurement at 260 nm (A_{260}) in a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies). The quality of the extracted RNA was evaluated from the absorbance ratio A_{260}: A_{280} (Sambrook & Russell 2001). The concentration of RNA was adjusted to 1 ng $µl^{-1}$ and used immediately or stored at –20°C until use.

Primers and probes

Before the experimental assays, an *in silico* test was performed to select primers and probes in order to detect any genotype of VHSV. With this in mind, a multiple sequence alignment was applied, using Clustal W, on a selection of strains from all genotypes of the virus (Table 1). Both G and N genes were subjected to analysis to find an optimum region to design

Table 1. Strains of viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), and infectious pancreatic necrosis virus (IPNV) used in this study. 1: Strains employed in the *in silico* assays; 2: strains employed in the sensitivity assays; 3: strains employed in the specificity assays

	Isolate	Assay	Host species	GenBank accession no.
VHSV strains (genotype)				
I	DK-F1	3	*Oncorhynchus mykiss*	
Ia	Fr0771	1, 2, 3	*Oncorhynchus mykiss*	AJ233396
	OM13.01	3	*Oncorhynchus mykiss*	
	Ssp11.02	3	*Solea* sp.	
	STF180.01	3	*Salmo trutta fario*	
	SS19.01	3	*Salmo salar*	
	CP4370	3	*Chondrostoma polylepis*	
Ib	DK5p31	2, 3	*Clupea harengus*	
	DK1p40	3	*Rhinonemus cimbrius*	
	DK1p12	3	*Clupea harengus*	
	DK4p37	1	*Micromesistius poutassou*	FJ460590
	SE-SVA-1033	1	*Oncorhynchus mykiss*	FJ460591
Id	NO-A163-68EG46	1	*Oncorhynchus mykiss*	AB672619
Ie	GE-1.2.	1	*Oncorhynchus mykiss*	AB672617
II	DK1p49	1, 2, 3	*Clupea harengus*	**KM244767**
	DK1p52	1, 3	*Sprattus sprattus*	AB672621
	DK1p53	3	*Clupea harengus*	
	DK1p54	3	*Gadus morhua*	
	DK5p551	3	*Clupea harengus*	
III	GH40	1, 3	*Reinhardtius hippoglossoides*	**KM244768**
	FA281107	1	*Oncorhynchus mykiss*	EU481506
	UK860/94	3	*Scophthalmus maximus*	
	SA2648	3	*Sparus aurata*	
	SC2645	3	*Oncorhynchus kisutch*	
	SM2705	3	*Scophthalmus maximus*	
	MLA98/6WH1	2, 3	*Merlangius merlangus*	
	DK4p101	3	*Merlangius merlangus*	
IVa	US-Makah	1, 2, 3	*Oncorhynchus kisutch*	X59241
	KRRV9822	1	*Paralichthys olivaceus*	AB179621
	US93EB5	3	*Clupea* sp.	
	JP99Obama25	3	*Paralichthys olivaceus*	
	JF00EhiI	1	*Paralichthys olivaceus*	AB490792.1
IVb	MI03GL	1	*Esox masquinongy*	GQ385941
	Goby1-5	1, 2, 3	*Neogobius* sp.	AB672615
IVc	CA-NB-00-01	1	*Fundulus heteroclitus*	EF079895
Non-specific reference strains				
IHNV	WRAC	1, 3	*Oncorhynchus mykiss*	AY442518
IHNV	LB91KI	1	*Oncorhynchus nerka*	AY438975
IHNV	River Butte 91	2, 3		
IHNV	Applegate River 93	2, 3		
IPNV	West Buxton	3		

a universal set of primers and probe, and the best one, at least at a predictable level, was observed in a conserved sequence of the N gene. Primers VHSV-TUsense (5′-CCG CGA GTT CGC YGA GCT TGT-3′) and VHSV-TUantisense (5′-GGR GCT CCT GAA GTT GCG GGC-3′) were designed using the Allele ID 3.0 software (Premier Biosoft) to amplify a fragment of 163 bp between positions 1149–1169 and 1311–1291 of the N gene of strain Fr0771 of VHSV (GenBank accession no. AJ233396). The same software was used to design the TaqMan probe TQM-TU (5′-ATC AGG GAG GCG GCC AGA CAG CA-3′). The probe, labelled at the 5′ end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3′ end with the Black Hole Quencher (BHQ1), was ordered from Sigma.

Reverse transcription

The synthesis of cDNA from viral RNA was performed using Superscript III RT (Invitrogen). To this end, 9 µl of extracted RNA were mixed with 2.5 ng μl^{-1} of random primers and the mixture was incu-

bated at 95°C for 5 min and on ice for at least 1 min. A reverse transcription mixture containing 10 U μl^{-1} of enzyme, 0.5 mM dNTPs and 0.05M DTT in 1× first strand buffer was then added, and the final mixture was incubated at 25°C for 10 min, followed by 50 min at 50°C. The reaction was finally stopped by heating at 85°C for 5 min.

SYBR Green qPCR

qPCR was performed using an iCycler iQ CFX96™ Real Time System (Bio Rad). The reactions were carried out in a final volume of 20 µl containing 100 nM of each primer set and 2 µl of cDNA in iQ™ SYBR® Green Supermix (BioRad). Following an initial 2 min activation/denaturation step at 95°C, the mixture was subjected to 40 cycles of amplification (denaturation for 15 s at 95°C, annealing and extension for 30 s at 58°C). Generation of PCR products was monitored after each extension step at 82°C by measuring the fluorescence of double-stranded DNA binding SYBR Green dye. In order to determine the melting temperatures (T_m) of the amplified products after SYBR Green qPCR, the temperature was raised from 58 to 95°C and the fluorescence detected during 10 s after each 0.2°C. From each reaction, the threshold cycle value (Ct) was established as the cycle number at which fluorescence was detectable over the threshold value calculated by the iCycler iQ software (Bio-Rad) for cycles 2–10.

TaqMan qPCR

qPCR was performed using the KAPA PROBE FAST universal qPCR Kit (Kapa Biosystems). Mixtures of a total volume of 20 µl contained 2 µl of cDNA, 10 µl of Kapa probe Fast Universal 2× qPCR Master Mix, 250 nM of probe and 100 nM of each primer. Following an initial 2 min activation/denaturation step at 95°C, the mixture was subjected to 40 cycles of amplification (denaturation for 15 s at 95°C, annealing and extension for 30 s at 58°C) in an iCycler iQ CFX96™ Real Time System (BioRad).

Evaluation of analytical sensitivity

The sensitivity of the diagnostic procedure was evaluated against different standards: titrated virus, RNA from crude virus, RNA from purified virus, plasmid DNA and *in vitro* transcribed RNA. Three replicates per assay and dilution (intra-run repeatability), and 3 different assays on 3 different days (inter-run reproducibility) were performed by workers with different levels of experience (with a minimum of 2 yr of training). In order to evaluate the reliability of the standards, and consequently of the detection limit obtained, a standard curve for each set of data was generated and a regression line calculated for the logarithm of the number of copies and the corresponding Ct values. The parameters considered to evaluate the reliability of the curve were the coefficient of determination (R^2) and the amplification efficiency (E) calculated from the formula $E = 10^{-1/S} - 1$ (where S is the slope of the linear fit).

Minimum detectable viral titre

To assess the minimum quantity of virus detectable by this qRT-PCR procedure, and to compare it with the sensitivity of the traditional method of isolation in cell culture, 1 reference strain from each genotype was tested: strain Fr0771 from genotype Ia, DK-5p31 from genotype Ib, DK-1p49 from genotype II, UK-MLA98/6WH1 from genotype III and strains US-Makah and Goby1-5 from genotypes IVa and IVb, respectively. For this assay, 10-fold dilutions of the corresponding virus were prepared and distributed into 2 aliquots: the first was subjected to qRT-PCR and the second to isolation in cell culture using monolayers of EPC cells.

Detection limit using RNA from crude and purified virus

Purification of virus was performed as previously described (Cutrín et al. 2009). Both crude and purified virus were subjected to total RNA extraction as described above. To prepare the RNA standards, the RNA concentration was adjusted to 1 ng μl^{-1} and serial 10-fold dilutions were prepared down to 1 ag μl^{-1}. All dilutions were subjected to qRT-PCR.

Detection limit using plasmid DNA

Specific primers for the nucleoprotein gene encompassing the regions corresponding to primer set VHS-TU (cm3a: CAG GCG TTG TCC GTG CTT CT; and N3b: CTA AAA GGC ACT CCC GTC TCA TAA) were designed to produce a fragment of 1049 bp from the FR07/71 strain. After RT-PCR, the amplicon

was purified and cloned into the pGEM-T Easy vector (Promega) under conditions recommended by the supplier, and individual clones were isolated. The cloned plasmid DNA was extracted and purified with the GeneJET™ Plasmid Miniprep kit (Fermentas). For quantification, the linearized plasmid and insert were run in an agarose gel (2%) with Trackit™ 1Kb Plus DNA Ladder (Invitrogen) as reference. Densitometry was applied on the gel, and quantification was performed with General-Purpose Analysis Software FUJI FILM Multi Gauge version 3.0 (Fuji Photo Film). The mass of a single pDNA molecule was calculated using the formula $\gamma = n/N \times GL \times NcMw$, where γ is the amount (in g), n is the number of DNA molecules (copies), N is the Avogadro number (6.022×10^{23}), GL is the size of the recombinant plasmid (4064 bp, with 3015 bp corresponding to pGEM-T and 1049 bp to the insert), and NcMw is the average molecular weight of each pair of nucleotides (estimated at 660 g mol^{-1}). The concentration of the resulting plasmid solution was around 200 ng μl^{-1} (corresponding to 4.49×10^{10} copies μl^{-1}), and 10-fold serial dilutions were prepared down to 1 ag μl^{-1} and subjected to qPCR.

Detection limit using an RNA transcript

Approximately 400 ng of the previously constructed plasmid were linearized by digestion with *Sal*I in a 20 µl reaction according to the enzyme manufacturer's instructions (FastDigest® SalI, Fermentas). The reaction was incubated for 15 min at 37°C, followed by heat inactivation at 65°C for 10 min. The linearized DNA was purified using the QIAquick PCR purification kit (Qiagen), according to the manufacturer's protocol. RNA transcripts were generated using the MEGAscript® Kit (Ambion). Approximately 0.5 µg of linearized plasmid were added to 20 µl of transcription reaction, containing 5 mM of each ribonucleotide and 2 µl of enzyme mix in 10× Reaction buffer. The transcription reaction was incubated at 37°C for 4 h. A DNase treatment was conducted using Ambion's Turbo DNase Kit for 30 min at 37°C. To recover the RNA, the MEGAclear™ Kit (Ambion) was used according to the manufacturer's instructions, and RNA transcripts were quantified using a Nanodrop ND-100. Copy numbers were then determined based on the expected N-gene transcript molecular weight, based on the formula $\gamma = n/N \times GL \times NcMw$, where γ is the amount (in g) of viral RNA, n is the number of genomic RNA molecules (copies), N is the Avogadro number (6.022×10^{23}), GL is the transcript length in nucleotides (1153 nt), and NcMw is the average molecular weight of a single nucleotide (estimated at 340.5 Da). From the formula, 1 µg of N transcript was deduced to contain 1.53×10^{12} copies. Serial dilutions were prepared down to 1 ag μl^{-1} and subjected to real-time RT-PCR.

Evaluation of specificity

To test specificity, a panel of 26 VHSV isolates covering the 4 genotypes was used (Table 1). In addition, 3 strains of IHNV type 1 (WRAC, River Butte 91 and Applegate River 93), a virus closely related to VHSV, and 1 strain of infectious pancreatic necrosis virus (IPNV; West Buxton) were included as negative controls. Additionally, to confirm the specificity of the amplified products, 3 methods were used: (1) in the SYBR Green assays, the melting temperature (T_m) was automatically calculated by the equipment, and the value obtained compared with that deduced from the sequence of the expected fragment; (2) the size of the amplicons was verified by electrophoresis in 1.5% agarose gels; (3) the amplified fragments were subjected to sequence analysis with the CEQ™ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter), after extraction of the DNA from the agarose gel with the DNA Gel Extraction Kit (Millipore).

Evaluation of repeatability and reproducibility

Repeatability (defined as the precision determined under conditions where the same methods and equipment are used by the same operator to make measurements on identical specimens) and reproducibility (the precision determined under conditions where the same methods but different equipment or days are used by different operators) were determined by the intra-assay and inter-assay (respectively) coefficients of variation (CV). Intra-run CV was determined with Ct values from 3 replicates of each reaction, and the inter-run CV was calculated with Ct values collected from 3 different reactions (runs) of the same samples performed on non-consecutive days.

Validation of the procedure for quantification

Once the reliability of the standard curves was demonstrated from the repeatability and repro-

ducibility, the determination coefficients and the efficiency values as described above, a general equation was obtained for each type of standard tested (viral titre, RNA from crude and from purified virus, RNA transcript and plasmid) and for each of both chemistries assayed (SYBR Green and TaqMan probes). Those equations were of the type $Ct = Sc + I$, where I is the intercept value, S is the curve slope, c is the number of copies assayed (as decimal logarithm), and Ct is the corresponding Ct value obtained.

In a diagnostic procedure, however, the focus is to determine the number of RNA copies corresponding to a Ct value observed after amplification of an RNA sample. Therefore, the initial equations were transformed by the isolation of the c variable into $c = S' Ct + I'$, where S' and I' are the recalculated slope and intercept values.

To determine whether 2 or more curves were similar, the slopes and intersection were compared by an F-test, using the SGPP5 software (Graph Pad Prism). If the p-value is small ($p < 0.05$) for both parameters, the idea that differences are due to random sampling can be rejected and therefore the curves are different; if differences are only demonstrated for the intersections, the curves are assumed to be parallel. To determine whether the use of different chemistries implies significant differences in the standard curves, the data yielding both equations for each type were compared by a t-test using Mann-Whitney with SGPP5; as before, significant differences were defined with p-values under 0.05.

RESULTS

Selection of primers and probe

To design a qRT-PCR assay for detection of any genotype of VHSV, the first step was the *in silico* selection of the best set of primers and probe, which were selected in a conserved region identified within the N gene of VHSV on the basis of 15 available sequences as shown in Fig. 1. Neither the primers nor the probe showed significant homology with the IHNV sequences tested as a negative control. In addition, NCBI resources and Allele ID 7.60 software (Premier Biosoft International) were used to ensure that this set of primers/probe did not recognise other related viruses or could amplify unwanted targets, e.g. in the fish genome (data not shown).

Finally, in order to determine their optimal concentration in the reaction mixtures, preliminary qRT-PCR reactions were performed with different con-

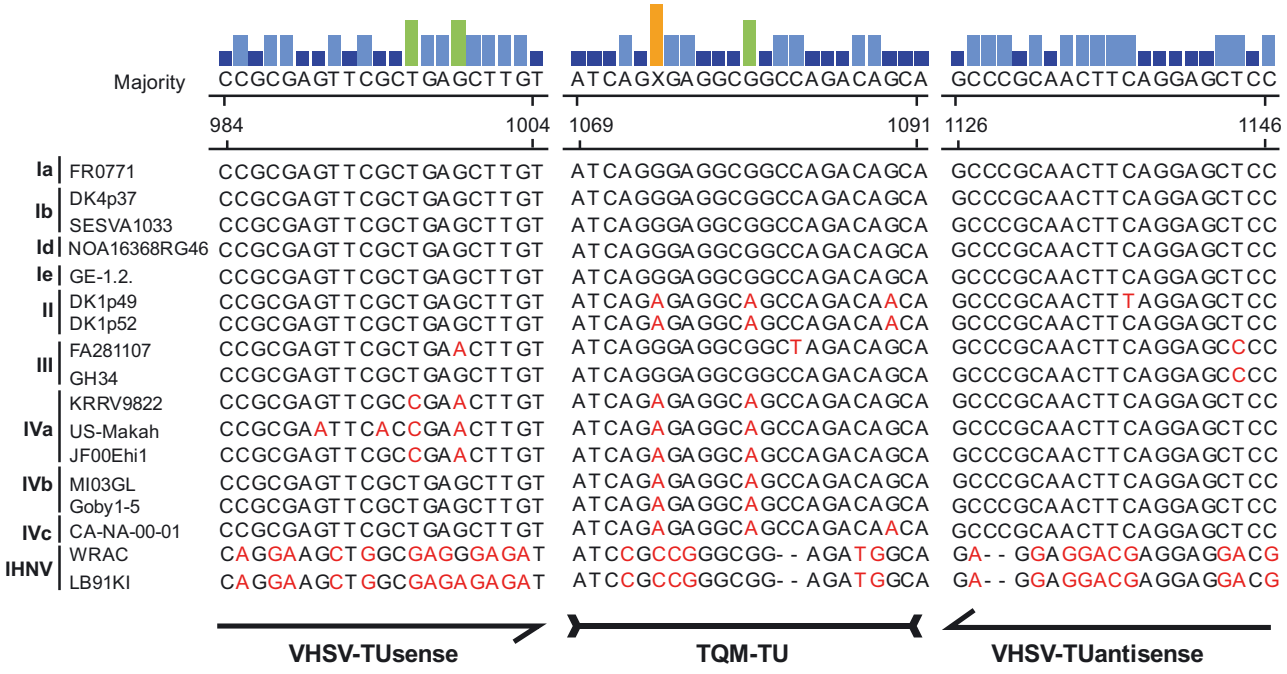

Fig. 1. Design of TaqMan probe and primers. Multialignment of partial sequences of the N-gene of different viral haemorrhagic septicaemia virus (VHSV) isolates showing the positions and sequences of 1 set of primers (VHSV-TU) and probe (TQM-TU) assayed. Accession numbers are shown in Table 1

centrations of primers and probe between 125 and 300 nM, and the results showed 250 nM to be the optimal concentration for both chemicals assayed in terms of Ct values and level of fluorescence (data not shown).

Analytical specificity

The specificity of the procedure was tested against a panel of 26 isolates, covering all VHSV genotypes, as well as several negative controls including IHNV and IPNV strains (Table 1). The procedure provided amplification with all of the VHSV strains tested, independently from their genotype, with an average Ct value of 15.32 ± 2.08 (Fig. 2A); in addition, no false positives were recorded when using nonspecific viruses as a negative control. To confirm the specificity of the amplicons, the melting point (T_m) was calculated automatically, and the observed mean values resembled those expected from the *in silico* theoretical calculation: 87.0 ± 0.1°C for Genotype I isolates (87. ± 0.1 *in silico* theoretical T_m), 85.4 ± 0.3°C for genotype II isolates (85.2 theoretical T_m), 86.5 ± 0.2°C for genotype III (86.7 ± 0.2 theoretical T_m) and 85.9 ± 0.2°C (86.2 ± 0.2 theoretical T_m) for genotype IV (Fig. 2B). The amplification products were subjected to agarose gel electrophoresis. As shown in Fig. 2C, the expected 163 bp PCR product was obtained in all cases (except with the non-specific negative control viruses), and sequencing confirmed their specificity.

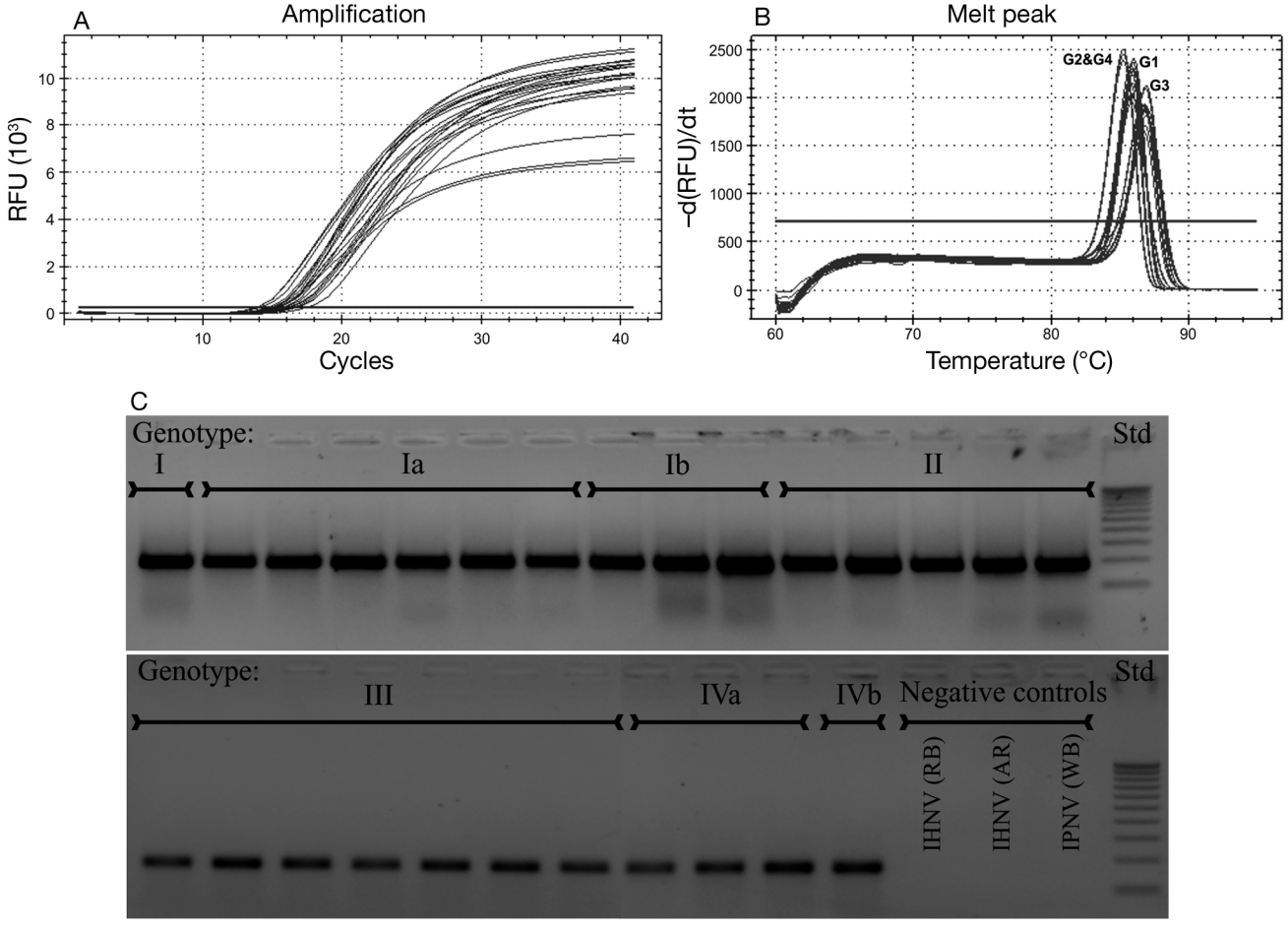

Fig. 2. Development of the SYBR Green qRT-PCR assay (RFU: relative fluorescence units), with primers TU-VHSV for the detection of viral haemorrhagic septicaemia virus (VHSV) isolates belonging to 4 genotypes. (A) Representative amplification profiles. (B) Representative melting temperature (T_m) analysis routinely performed on qRT-PCR products. (C) Visualization of the amplification products after agarose gel electrophoresis. For all VHSV strains, the visualized band corresponded to an amplification fragment of the expected size of 163 bp. Two strains of infectious haematopoietic necrosis virus (IHNV: River Butte 91 and Applegate River93) and 1 strain of infectious pancreatic necrosis virus (IPNV: West Buxton) were used as negative controls. Std: 100 bp DNA ladder (10 fragments ranging from 100 to 1000 bp in 100 bp increments; Genecraft)

Analytical sensitivity

Minimum detectable viral titre

A comparison of the limit of detection (LD), in terms of minimum detectable viral titre, between qRT-PCR and isolation in cell culture was performed, and the results are shown in Table 2. As observed, the LD was 2 logs lower with qRT-PCR than with cell culture isolation (where all 3 replicates were positive) for most of the reference strains. No differences between the chemistries (SYBR Green and TaqMan probe) were observed, ensuring an LD, in terms of viral titre, of around 10^0 $TCID_5$ ml^{-1} with most genotypes (close to 10^{-1} with DK1p49 and Goby1-5).

The qRT-PCR results from these assays were also used to evaluate the reliability of this kind of standard curve. The results shown in Table 2 correspond to the mean (± SD) from the 3 runs performed on different days. Previously, the analysis of the CV values from the 3 replicates of each run revealed a high level of repeatability, obtaining CV values from 0.1 to 1.4 (data not shown). Reproducibility is deduced from the data shown in the table, which provide CV values between 0.1 and 2.4. Linear regression was applied to the data, and the standard curves with both SYBR Green and TaqMan chemistries are shown in Fig. 3. All the standard curves showed high R^2 values, ranging between 0.990 and 0.995, and efficiencies (E) from 104% (slope = −3.221) to 95% (slope = −3.442).

Table 2. Comparison of the limit of detection (in terms of viral titre) of viral haemorrhagic septicaemia virus (VHSV) between qRT-PCR and cell culture isolation. CC: viral detection by isolation in epithelioma papulosum cyprini (EPC) cells from serial dilution of crude virus. Dil: 10-fold serial dilutions were performed to obtain the indicated viral titres (in $TCID_{50}$ ml^{-1}). The number of pluses indicates the number of positives among the 3 replicates (dilutions showing positive in the 3 replicates yielded the same result in the 3 runs). qRT-PCR/SYBR: viral detection by SYBR Green qRT-PCR using primers VHSV-TU; qRT-PCR/TQM: viral detection by qRT-PCR using primers VHSV-TU and TaqMan probe TQM-TU. Values shown are the threshold cycle value (Ct) ± SD of 3 repetitions. **Bold** font indicates that only 1 of 3 replicates was amplified

Fr0771								
Dil	**10^6**	**10^5**	**10^4**	**10^3**	**10^2**	**10^1**	**10^0**	**10^{-1}**
CC	+++	+++	+++	+++	+++	+	+	
qRT-PCR/SYBR	14.2±0.06	18.5±0.13	22.1±0.06	25.9±0.03	28.6±0.22	31.5±0.16	34.2±0.31	
qRT-PCR/TQM	14.4±0.08	18.6±0.12	22.2±0.04	25.9±0.05	28.6±0.19	31.2±0.11	34.2±0.19	**38.1±22.0**
DK5p31								
Dil	**10^6**	**10^5**	**10^4**	**10^3**	**10^2**	**10^1**	**10^0**	
CC	+++	+++	+++	+++	+++	+	+	
qRT-PCR/SYBR	14.4±0.13	18.6±0.14	22.3±0.09	25.5±0.11	28.4±0.09	31.3±0.08	34.2±0.30	
qRT-PCR/TQM	14.8±0.05	18.5±0.45	22.3±0.40	25.9±0.10	28.2±0.03	31.2±0.05	34.5±0.15	
DK1p49								
Dil	**5.6×10^5**	**5.6×10^4**	**5.6×10^3**	**5.6×10^2**	**5.6×10^1**	**5.6×10^0**	**5.6×10^{-1}**	
CC	+++	+++	+++	+++	+++	+		
qRT-PCR/SYBR	15.1±0.06	19.1±0.07	23.5±0.10	26.5±0.11	29.5±0.06	32.9±0.53	35.6±0.23	
qRT-PCR/TQM	15.1±0.10	19.0±0.11	23.2±0.46	26.4±0.09	29.6±0.05	32.5±0.10	35.5±0.29	
MLA98/6WH1								
Dil	**10^5**	**10^4**	**10^3**	**10^2**	**10^1**	**10^0**	**10^{-1}**	
CC	+++	+++	+++	+++	+	+		
qRT-PCR/SYBR	18.6±0.14	22.6±0.44	25.2±0.21	28.4±0.46	31.6±0.23	34.2±0.07		
qRT-PCR/TQM	18.6±0.15	22.3±0.38	25.4±0.13	28.5±0.29	31.5±0.44	34.6±0.17		
US-Makah								
Dil	**5.6×10^5**	**5.6×10^4**	**5.6×10^3**	**5.6×10^2**	**5.6×10^1**	**5.6×10^0**	**5.6×10^{-1}**	
CC	+++	+++	+++	+++	+++	+		
qRT-PCR/SYBR	15.1±0.23	19.4±0.12	22.7±0.15	25.2±0.07	28.8±0.36	32.9±0.32		
qRT-PCR/TQM	15.2±0.33	19.3±0.29	22.5±0.25	25.3±0.42	28.4±0.20	33.1±0.19	**36.2±22.0**	
Goby1-5								
Dil	**5.6×10^5**	**5.6×10^4**	**5.6×10^3**	**5.6×10^2**	**5.6×10^1**	**5.6×10^0**	**5.6×10^{-1}**	
CC	+++	+++	+++	+++	+++	+		
qRT-PCR/SYBR	15.2±0.08	19.0±0.43	23.1±0.14	25.5±0.13	29.4±0.25	33.3±0.32	35.4±0.38	
qRT-PCR/TQM	15.0±0.20	19.5±0.35	23.0±0.29	25.5±0.18	29.2±0.14	33.3±0.34	35.4±0.43	

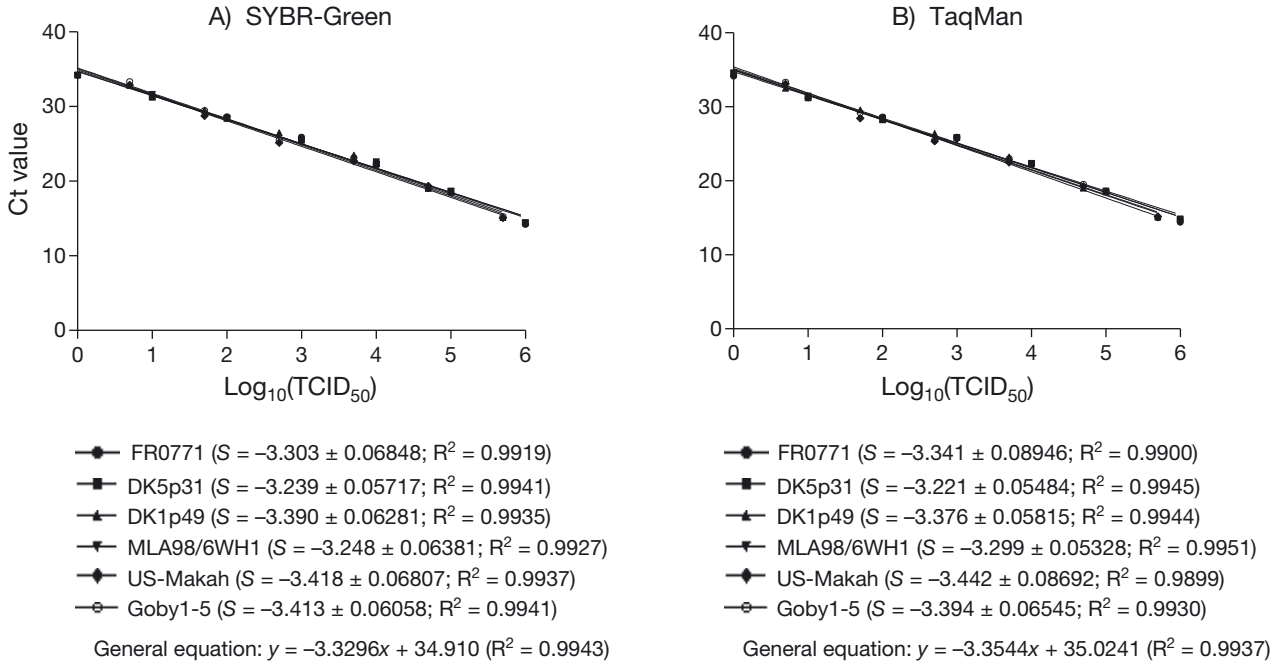

Fig. 3. Standard curves of minimum detectable viral titre. RNA extracted from serial dilutions of crude virus from all genotypes of viral haemorrhagic septicaemia virus (VHSV) was used as a standard for detection by (A) SYBR Green and (B) TaqMan RT-real time PCR. Graphs show linear regression data from the 3 replicates and 3 repetitions (*S*: slope expressed as mean ± SD; R^2: average determination coefficient). The general equation is a single equation summarizing the results obtained from 3 replicates and 3 repetitions using all the strains assayed. See 'Materials and methods' for further details

In addition, the comparison of the slopes and intercepts (within each type of chemistry) revealed no significant differences ($p > 0.05$) between strains. Therefore, a unique equation was calculated summarizing the results obtained from the 3 replicas and 3 repetitions with all strains assayed, providing slopes of –3.329 (E = 99%) with SYBR Green, and –3.354 (E = 98%) with TaqMan probes, and R^2 values of 0.9943 and 0.9937, respectively.

LD of viral RNA from crude and purified virus

Sensitivity was also evaluated with RNA extracted from purified virus (strain Fr0771, belonging to genotype Ia). As shown in Table 3, the LD reached 100 ag (16 genome copies), at a Ct value between 35 and 36, with both chemistries. The data shown in Table 3 correspond to the mean (± SD) from the 3 repetitions performed on different days. Previously, the analysis of the CV values from the 3 replicates revealed a high level of repeatability, obtaining CV values from 0.3 to 1.3 with SYBR Green, and from 0.1 to 1.2 with TaqMan (data not shown). The CV values corresponding to reproducibility were extremely low (from 0.43 to 1.49% with SYBR Green and from 0.60 to 1.24% with TaqMan; Table 1). The standard curves showed high R^2 levels (from 0.997 to 0.998) and efficiency values (between 109% and 104%, as determined from the slopes; Fig. 4). The slopes and intercepts were compared, and no significant differences were observed ($p > 0.05$). Therefore, a unique equation, with high R^2 (0.998 and 0.997, SYBR Green and TaqMan, respectively) and E (104% and 108%) values, was calculated for each chemistry from the 3 replicates and repetitions (Fig. 4).

This assay was also performed using RNA extracted from crude virus of a representative strain of each genotype and, as shown in Table 3, the procedure yielded similar results with both chemistries, achieving the detection of viral RNA to a minimum concentration of 1 fg μl^{-1} (corresponding to approximately 160 genome copies), and without differences between genotypes. All replicates yielded similar results, with low variations in Ct values and low coefficients of variation (under 2.98), corresponding to high repeatability (data not shown). As shown in the table, inter-run CV values (reproducibility) were also low (0.69–3.02% with SYBR Green; 0.14–3.12% with TaqMan). Linear regression was applied to the data

Table 3. Limits of detection of viral RNA from purified and crude viral haemorrhagic septicaemia virus (VHSV) by SYBR Green and TaqMan-Probe qRT-PCR. The assay using RNA extracted from purified virus was performed with strain Fr0771. The assay using RNA extracted from crude virus was performed with a reference strain from each genotype. No. of copies: corresponding number of genome copies, calculated from the formula $\gamma = n/N \times GL \times NcMw$ as described in the 'Materials and methods'; RNA: quantity of extracted RNA employed for detection by qRT-PCR. Values shown are mean threshold cycle values (Ct from 3 runs ± SD), and the corresponding CVs are given as percentages; ND: not detected

No. of copies	RNA	Purified virus Fr0771 (Ia)		Crude virus											
				FR0771 (Ia)		DK5p31 (Ib)		DK1p49 (II)		MLA98/6WH1 (III)		US-Makah (IVa)		Goby1-5 (IVb)	
		Ct	CV	Ct	CV	Ct	CV	Ct	CV	Ct	CV	Ct	CV	Ct	CV
(A) SYBR-Green															
1.6×10^{8}	1 ng	13.3±0.20	1.49	14.0±0.40	2.87	14.3±0.29	1.99	14.0±0.35	2.53	14.1±0.18	1.24	13.9±0.18	1.26	14.5±0.33	2.30
1.6×10^{7}	100 pg	16.2±0.19	1.19	17.1±0.46	2.69	17.2±0.26	1.50	16.9±0.27	1.63	17.4±0.28	1.63	17.5±0.19	1.11	17.1±0.44	2.59
1.6×10^{6}	10 pg	19.4±0.18	0.93	22.0±0.46	2.10	22.5±0.18	0.82	22.0±0.30	1.37	22.0±0.28	1.25	22.3±0.43	1.91	21.7±0.47	2.17
1.6×10^{5}	1 pg	22.3±0.10	0.43	25.8±0.32	1.23	25.2±0.44	1.76	25.1±0.41	1.64	25.1±0.21	0.85	25.4±0.33	1.29	25.2±0.33	1.29
1.6×10^{4}	100 fg	25.3±0.18	0.70	29.1±0.48	1.67	29.6±0.28	0.95	29.0±0.36	1.25	28.9±0.43	1.50	29.7±0.90	3.02	28.9±0.20	0.69
1.6×10^{3}	10 fg	28.9±0.14	0.47	32.1±0.54	1.68	32.3±0.34	1.04	31.9±0.42	1.31	31.8±0.30	0.94	32.3±0.43	1.34	31.7±0.35	1.10
1.6×10^{2}	1 fg	32.4±0.27	0.83	34.5±0.91	2.65	35.1±0.47	1.35	34.0±0.50	1.48	34.2±0.47	1.38	35.0±0.43	1.23	34.3±0.43	1.25
1.6×10^{1}	100 ag	35.8±0.15	0.43	ND		ND		ND		ND		ND		ND	
(B) TaqMan probe															
1.6×10^{8}	1 ng	13.2±0.16	1.24	14.3±0.20	1.37	14.2±0.13	0.91	13.9±0.37	2.66	14.5±0.31	2.14	14.5±0.45	3.12	14.4±0.21	1.46
1.6×10^{7}	100 pg	16.3±0.18	1.12	17.1±0.22	1.28	17.4±0.09	0.51	17.6±0.13	0.72	17.4±0.29	1.64	17.7±0.41	2.34	17.5±0.35	2.01
1.6×10^{6}	10 pg	20.3±0.15	0.74	22.3±0.35	1.58	22.3±0.19	0.86	22.1±0.32	1.45	22.2±0.48	2.16	22.3±0.36	1.60	21.9±0.23	1.04
1.6×10^{5}	1 pg	23.0±0.14	0.60	25.3±0.20	0.79	26.0±0.09	0.33	25.7±0.34	1.33	25.4±0.20	0.80	25.6±0.08	0.30	25.1±0.17	0.66
1.6×10^{4}	100 fg	26.0±0.20	0.75	28.9±0.31	1.09	29.2±0.44	1.51	28.7±0.18	0.63	28.1±0.15	0.55	29.1±0.08	0.29	29.0±0.11	0.37
1.6×10^{3}	10 fg	29.2±0.35	1.18	32.0±0.23	0.70	32.7±0.45	1.37	32.4±0.33	1.00	31.9±0.26	0.83	32.1±0.18	0.57	31.7±0.27	0.84
1.6×10^{2}	1 fg	32.1±0.37	1.14	35.4±0.35	0.98	34.8±0.69	1.97	34.7±0.33	0.95	34.8±0.18	0.52	35.0±0.05	0.14	33.9±0.27	0.80
1.6×10^{1}	100 ag	35.3±0.29	0.82	ND		ND		ND		ND		ND		ND	

(Fig. 5), and the standard curves for all the strains showed R^2 values between 0.991 and 0.997. The slopes and intercepts were compared with the *F*-test, revealing no significant differences ($p > 0.05$) between strains. A unique equation for each chemistry was therefore calculated applying a new linear regression to all of the data, which yielded standard curves with high R^2 values (0.991 and 0.993, with SYBR Green and TaqMan, respectively) and efficiencies (E = 93.4%, S = −3.492 with SYBR Green; E = 94.1%, S = −3.475 with TaqMan).

LD using plasmid DNA as a standard

A fourth method to evaluate the sensitivity used a cloned cDNA corresponding to a fragment of the N gene of strain Fr0771 as a standard. Ten-fold dilutions were prepared from 2.25×10^{8} to 2.25×10^{-1} copies, and the LD was determined by both SYBR Green and TaqMan real-time PCR. Although the viral genome could occasionally be detected at the lowest concentration, in 1 out of 3 replicates (Table 4), the detection limit was established at 10 ag μl^{-1} of recombinant plasmid (corresponding to around 2 copies) with both SYBR Green and TaqMan, since the maximum reliability of the method (detection in all replicates) is required. Another remarkable result comes from the visualization of the amplification curves, since their kinetics seem to be proportionally related to the initial quantity of template (Fig. 6A,B); this was demonstrated by the high values of the corresponding determination coefficients (R^2) (Fig. 6C,D).

Intra-assay CV values (repeatability) were under 5 in all cases, and inter-assay CV (reproducibility) ranged from 0.15 to 4.47% with SYBR Green, and from 0.50 to 2.09% with TaqMan (Table 4). Linear regression was applied to the data from the 3 runs, yielding R^2 values between 0.990 and 0.997, and efficiencies (deduced from the slopes) be-

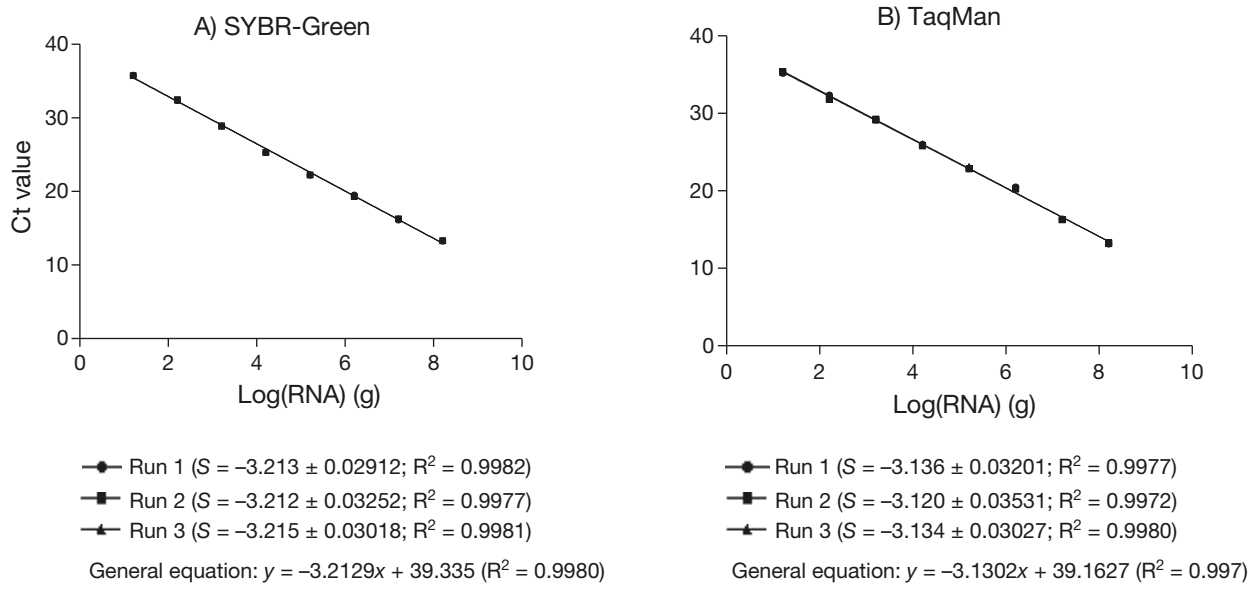

Fig. 4. Standard curves with purified virus. Serial dilutions of viral RNA extracted from purified viral haemorrhagic septicaemia virus (VHSV, strain FR0771) were used as a standard for detection by (A) SYBR Green and (B) TaqMan real-time RT-PCR. Graphs show 3 curves corresponding to 3 runs on different days (3 replicates per run). Equations as in Fig. 3

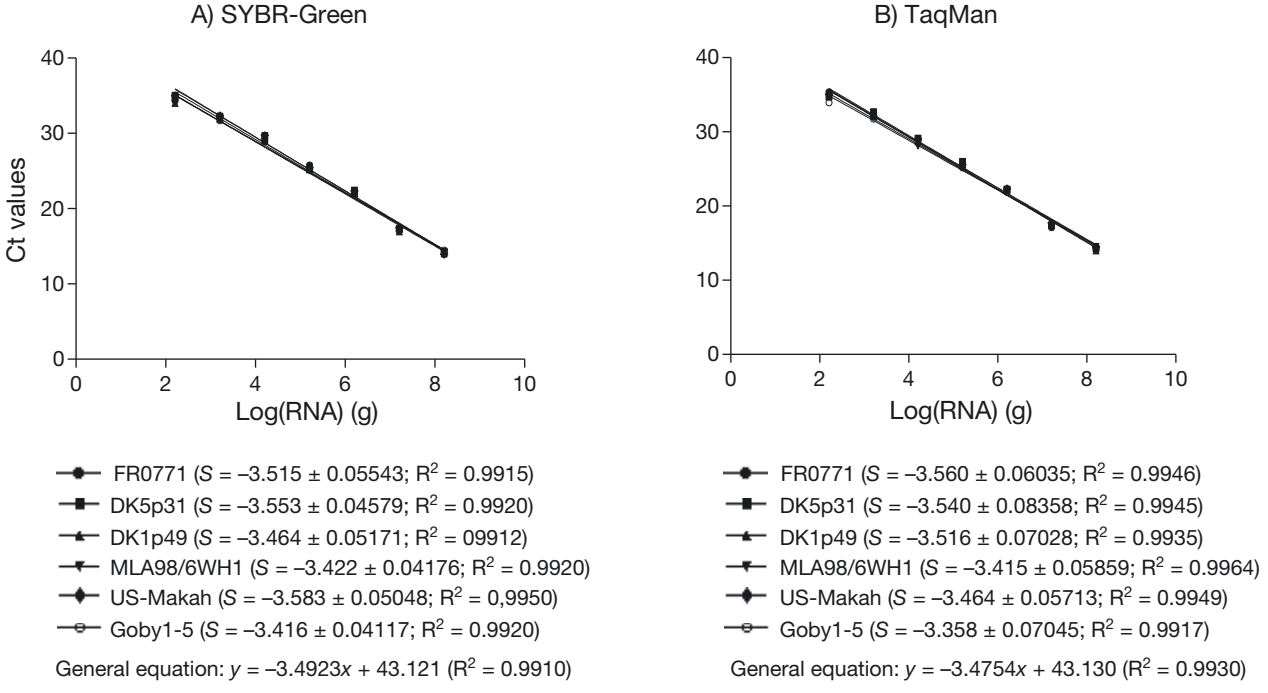

Fig. 5. Standard curves with crude virus. Serial dilutions of viral RNA extracted from crude virus of all genotypes of viral haemorrhagic septicaemia virus (VHSV) were used as a standard for detection by (A) SYBR Green and (B) TaqMan real-time RT-PCR. Equations as in Fig. 3

tween 93.4 and 104.7% (Fig. 6C,D). In addition, no significant differences between slopes and intercepts were observed ($p > 0.05$); therefore, a linear regression was applied to all of the data to obtain a new single standard curve for each chemistry, showing in both cases high R^2 (0.999 and 0.996, with SYBR Green and TaqMan, respectively) and efficiencies of 96.8 and 104.9% (Fig. 6C,D).

Table 4. Limit of detection of viral haemorrhagic septicaemia virus (VHSV) using plasmid DNA as standard. Details and abbreviations as in Table 3. [a]One of the 3 replicates was amplified

No. of copies	RNA	Run1 Ct	Run1 CV	Run2 Ct	Run2 CV	Run3 Ct	Run3 CV	Inter-run Ct	Inter-run CV
(A) SYBR-Green									
2.25×10^{8}	1 ng	8.5±0.36	4.25	8.3±0.06	0.72	8.1±0.13	1.61	8.3±0.26	3.11
2.25×10^{7}	100 pg	13.2±0.43	3.26	13.3±0.44	3.30	12.4±0.35	2.83	13.0±0.58	4.47
2.25×10^{6}	10 pg	16.3±0.07	0.42	16.9±0.07	0.41	15.6±0.09	0.58	16.3±0.61	3.77
2.25×10^{5}	1 pg	19. 7±0.08	0.40	20.5±0.27	1.32	18.8±0.14	0.74	19.7±0.72	3.66
2.25×10^{4}	100 fg	23.3±0.43	1.84	23.8±0.11	0.46	23.0±0.14	0.61	23.4±0.44	1.9
2.25×10^{3}	10 fg	26.6±0.07	0.26	27.3±0.12	0.44	26.5±0.04	0.15	26.8±0.35	1.32
2.25×10^{2}	1 fg	29.9±0.19	0.63	30.8±0.42	1.36	30.5±0.46	1.51	30.4±0.53	1.74
2.25×10^{1}	100 ag	33.3±0.57	1.71	33.3±0.20	0.60	33.0±0.29	0.88	33.2±0.37	1.1
2.25×10^{0}	10 ag	35.2±0.90	2.55	35.2±0.37	1.05	35.6±0.21	0.59	35.3±0.53	1.49
2.25×10^{-1}	1 ag	ND		ND		ND			
(B) TaqMan probe									
2.25×10^{6}	10 pg	17.4±0.07	0.38	17.7±0.09	0.49	17.5±0.09	0.51	17.5±0.19	1.08
2.25×10^{5}	1 pg	20.2±0.08	0.38	20.4±0.08	0.39	20.3±0.07	0.34	20.3±0.16	0.79
2.25×10^{4}	100 fg	22.8±0.43	1.88	23.0±0.41	1.77	22.8±0.31	0.13	22.9±0.41	1.81
2.25×10^{3}	10 fg	26.9±0.08	0.30	27.2±0.06	0.22	27.1±0.10	0.36	27.0±0.17	0.63
2.25×10^{2}	1 fg	30.2±0.08	0.25	30.5±0.10	0.32	30.4±0.10	0.32	30.4±0.18	0.59
2.25×10^{1}	100 ag	33.9±0.09	0.27	34.2±0.07	0.21	34.1±0.12	0.35	34.1±0.17	0.50
2.25×10^{0}	10 ag	35.9±0.64	1.77	36.8±0.70	1.91	36.9±0.50	1.3	36.4±0.76	2.09
2.25×10^{-1}	1 ag	38.8[a]		39.1[a]		39.2[a]		39.1±19.54[a]	49.90

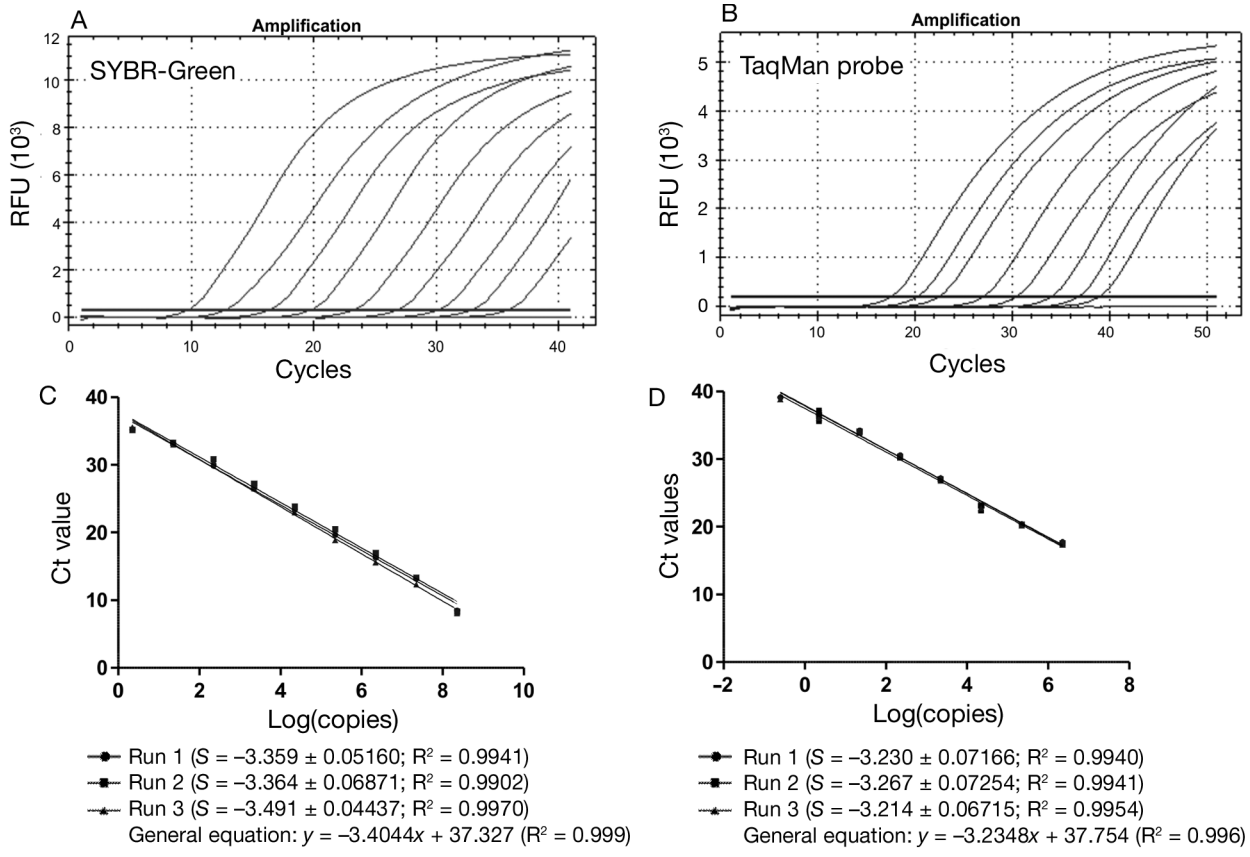

Fig. 6. Evaluation of analytical sensitivity using plasmid DNA as a standard. Ten-fold dilutions (from 2.2×10^{8} to 2.2×10^{-1} copies) of a cloned cDNA corresponding to a fragment of the N gene of viral haemorrhagic septicaemia virus (VHSV) strain Fr0771 were subjected to amplification by real-time PCR using (A,C) SYBR Green or (B,D) TaqMan probes. Three replicates per run and 3 runs (repetitions on different days) were performed. (A,B) Typical amplification curves for the serial concentrations of template; (C,D) linear regressions for the 3 runs. The general equation is a single equation summarizing the results obtained in the 3 replicates and 3 repetitions

Table 5. Limit of detection of viral haemorrhagic septicaemia virus (VHSV) using RNA transcript as standard. Details and abbreviations as in Table 3

No. of copies	RNA	Run1		Run2		Run3		Inter-run	
		Ct	CV	Ct	CV	Ct	CV	Ct	CV
(A) SYBR-Green									
1.53×10^{8}	100 pg	12.8±0.37	2.89	12.7±0.25	1.92	13.2±0.17	1.32	12.9±0.30	2.36
1.53×10^{7}	10 pg	16.2±0.38	2.37	16.6±0.45	2.73	16.3±0.35	2.15	16.4±0.39	2.35
1.53×10^{6}	1 pg	19.9±0.55	2.73	19.9±0.23	1.16	20.4±0.21	1.01	20.1±0.38	1.90
1.53×10^{5}	100 fg	24.0±0.63	2.65	23.9±0.10	0.42	24.5±0.11	0.44	24.1±0.42	1.74
1.53×10^{4}	10 fg	27.0±0.44	1.63	26.9±0.53	1.97	26.4±0.13	0.50	26.8±0.45	1.70
1.53×10^{3}	1 fg	31.1±0.26	0.84	30.6±0.30	0.98	30.9±0.40	1.28	30.9±0.36	1.17
1.53×10^{2}	100 ag	34.4±0.28	0.81	34.7±0.19	0.54	34.8±0.41	1.18	34.6±0.31	0.90
1.53×10^{1}	10 ag	37.2±0.16	0.42	37.4±0.20	0.52	37.0±0.33	0.88	37.2±0.25	0.68
1.53×10^{0}	1 ag	ND		ND		ND			
(B) TaqMan probe									
1.53×10^{8}	100 pg	12.8±0.36	2.79	11.6±0.43	3.71	12.2±0.42	3.45	12.2±0.62	4.88
1.53×10^{7}	10 pg	16.5±0.32	1.92	17.1±0.68	3.99	17.6±0.19	1.08	17.1±0.60	3.54
1.53×10^{6}	1 pg	20.2±0.07	0.35	19.6±0.33	1.67	19.5±0.31	1.57	19.8±0.43	2.18
1.53×10^{5}	100 fg	24.2±0.32	1.33	23.9±0.31	1.28	23.9±0.52	2.20	24.0±0.37	1.55
1.53×10^{4}	10 fg	26.9±0.30	1.11	26.6±0.43	1.62	26.6±0.36	1.34	26.7±0.36	1.34
1.53×10^{3}	1 fg	31.6±0.31	0.98	31.4±0.21	0.67	31.4±0.13	0.41	31.4±0.22	0.70
1.53×10^{2}	100 ag	34.3±0.28	0.82	34.1±0.27	0.81	33.7±0.43	1.28	34.0±0.40	1.16
1.53×10^{1}	10 ag	36.4±0.94	2.57	36.6±0.11	0.31	36.4±0.56	1.53	36.5±0.55	1.52
1.53×10^{0}	1 ag	ND		ND		ND			

LD using RNA transcript as a standard

An RNA transcript was constructed based on the same sequence used for the plasmid DNA. Ten-fold serial dilutions (from 100 pg μl^{-1} to 1 ag μl^{-1}, corresponding to 1.53×10^{8} to 1.53×10^{0} copies) were subjected to amplification by SYBR Green and TaqMan real-time RT-PCR, and the results are shown in Table 5 and Fig. 7. Detection of RNA transcript was achieved to a minimum concentration of 10 ag μl^{-1} (around 15 RNA copies) with both procedures. The intra-run CV values (repeatability) were below 2.9% with SYBR Green and 4.0% with TaqMan, and inter-run values (reproducibility) were below 2.4 and 4.9%, respectively (Table 5). As with pDNA, using RNA transcript as a standard, the amplification curves seemed to be proportionally distributed according to the initial quantity of template (Fig. 7A,B), which was demonstrated by the high R^2 values observed (Fig. 7C,D). In addition, no significant differences ($p > 0.05$) were observed between the slopes and intersections of the different runs. Linear regression applied to data from the 3 replicates and runs yielded standard curves with high R^2 (0.998 and 0.995, with SYBR Green and TaqMan, respectively) and efficiency values (92.3 and 94.1%, respectively; Fig. 7C,D).

Comparison of the standards for quantification

Once a single curve for each standard was obtained, and their reliability demonstrated, the next step was to evaluate whether significant differences were observed between both chemistries. To this end, a Mann-Whitney *t*-test was applied, demonstrating no significant differences ($p > 0.05$) between the equations of both chemistries, with any of the standards tested. Therefore, a unique equation for each standard was constructed using all available data from both chemistries.

To be used for quantification, those equations (of the type $Ct = Sc + I$) were transformed to $c = S'Ct + I'$ (as described in 'Materials and methods: Validation of the procedure …'), and the corresponding curves are shown in Fig. 8. Estimation of the quantity of virus clearly depended on the type of standard used as a reference, with the following scale (from lower to higher values): TCID, pDNA, purified virus, RNA transcript and crude virus. In addition, a certain parallelism appeared to exist among most of the curves, except with purified virus as a standard, which intersects (at low Ct values or high copy numbers) with the one corresponding to RNA transcript. To statistically determine which of those curves were truly parallel, the slopes of each pair of

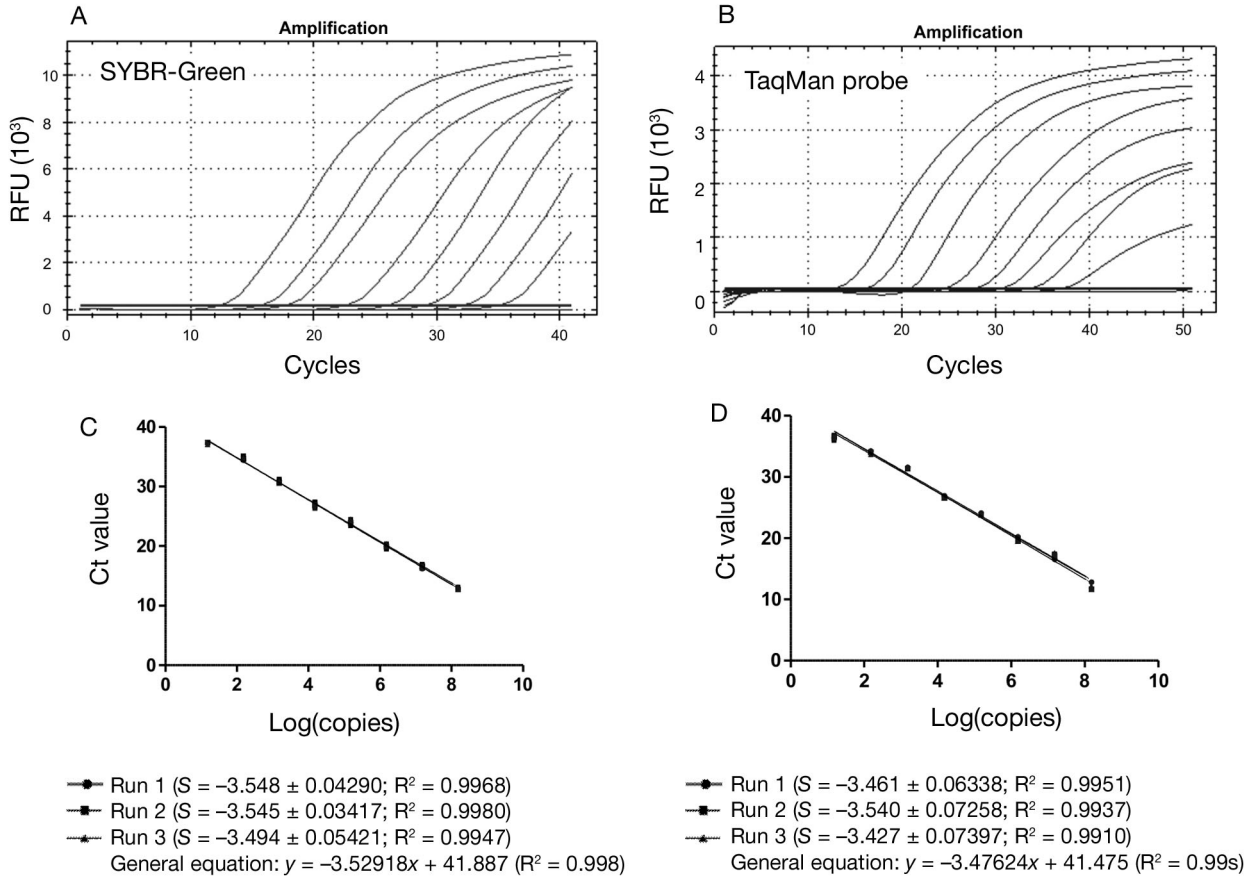

Fig. 7. Evaluation of analytical sensitivity using transcript RNA as a standard. Ten-fold dilutions (from 1.5×10^8 to 1.5×10^0 copies) of the transcript were subjected to amplification by real-time PCR using (A,C) SYBR Green or (B,D) TaqMan probes. Details as in Fig. 6

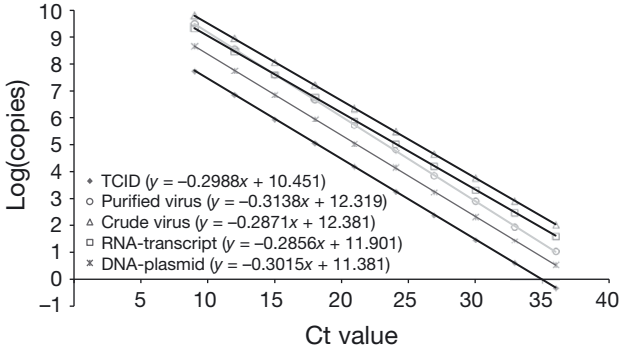

Fig. 8. Standard curves for viral quantification of viral haemorrhagic septicaemia virus (VHSV) using different types of template as standard

curves were compared and, in all cases, including pairs with the equation with purified virus, p was >0.05, demonstrating that all curves were parallel. After this surprising result, and to confirm that a unique conversion factor could be established between standards, the conversion factors (CF; comparing the expected number of copies from each pair of equations) between each set of 2 standard curves were calculated for Ct values between 9 and 36, and, as shown in Table 6, the ranges of CF values were in all cases under 1 logarithm of difference. Therefore, the statistical average CF is shown as the reference for comparison between standards of quantification.

DISCUSSION

At present, real-time PCR technology is replacing traditional PCR, not only because of its higher reliability in terms of sensitivity and specificity, but also because it provides quick and simple processing and interpretation of results (regardless of the type of chemistry used: SYBR Green or TaqMan probes), and allows relative or absolute quantification of the

Table 6. Mean conversion factors (CF) between each set of 2 standard curves (for threshold cycle values 9–36). Ranges of CF are given in parentheses

	Transcript	Purified virus	Plasmid	TCID
Crude virus	2.4 (2.31–2.53)	4.5 (1.7–9.1)	18.9 (11.6–28.6)	138 (94–194)
Transcript	1	0.7 (0.2–1.4)	7.9 (4.6–12.3)	57 (37–84)
Purified virus		1	4.7 (3.1–6.7)	35 (21–54)
Plasmid			1	7.4 (6.8–8.0)

template, which provides relevant information in a diagnosis. Therefore, the aim of the present study was to design and validate an qRT-PCR procedure for the diagnosis of VHSV of any genotype, which could be additionally and simultaneously employed to quantify the viral load.

Cutrín et al. (2009) reported a reliable multiplex real-time RT-PCR procedure for the diagnosis of VHSV, but it needed 2 sets of primers to ensure the detection of strains from the 4 genotypes and subtypes. Matejusova et al. (2008) designed a procedure with a single set of primers, which they called 'universal'; however, it was only tested on European strains. Therefore, in the present study, we decided to find a unique and truly universal set of primers and probe, and with this in mind, an *in silico* assessment was carried out on VHSV strains from different genotypes and subtypes, and the selection was performed in a consensus sequence targeting the N gene.

The *in vitro* evaluation of the primers (TU-VHSV), performed on 26 reference strains (10, 5, 7 and 4 strains from genotypes I, II, III and IV, respectively), demonstrated that they ensured detection of any type of strain, yielding amplicons of the correct size and sequence and, moreover, yielding the expected Tm values in the melting curves. No unspecific amplifications were observed with either unrelated or related viruses. Other studies have described the development of real-time RT-PCR for the diagnosis of this virus (Chico et al. 2006, Liu et al. 2008, Matejusova et al. 2008, Cutrín et al. 2009, Hope et al. 2010, Garver et al. 2011, Jonstrup et al. 2013, Pierce et al. 2013), but the detection of strains from all genotypes was not always ensured, or the procedure was not tested on a significant number of strains from each genotype. Two studies reported the testing of a procedure on a number of strains similar to that in the present study. Garver et al. (2011) demonstrated that the procedure they proposed ensured detection of all strains assayed (in the form of crude virus); however, they obtained Ct values ranging from 15.04 to 38.66 (21.83 ± 5.98), much higher that the Ct values observed in the similar experiment in our study (15.32 ± 2.08). Pierce et al. (2013) tested the specificity of their procedure on 25 strains of the 4 genotypes, with positive results in all cases; unfortunately, they did not provide information on the Ct values, which is important data in order to have an idea of the efficiency of the method for each type of strain.

As expected, the LD obtained was different depending on the type of reference employed to assess the sensitivity of the procedure (Table 7). In terms of minimum detectable viral titre, although some strains were detectable at a titre of 10^{-1} $TCID_{50}$ ml^{-1}, the LD was established at 10^0, since it ensures the detection of any type of strain (at a Ct around 36), and a dynamic range of 8 orders of magnitude. This LD improves that reported by Jonstrup et al. (2013), who obtained an LD (in BF-2 cells) of 10^1 to 10^2 $TCID_{50}$ ml^{-1}, and at least equals that reported by Hope et al. (2010). Furthermore, in our study, the LD is 100 times lower than that obtained with the traditional cell culture isolation, which is consistent with the results of both reports.

Using pDNA as the standard, the LD was quite similar to that of TCID: 2 copies (equivalent to 2 particles), at a Ct value of 37. The effective dynamic range was of 9 orders of magnitude: from 2 to 2.25×10^8 copies (Ct from 37 to 8). These results are similar to those reported for other viruses (Wang et al. 2006, Zhang et al. 2006), and improve those reported previously for this virus (Cutrín et al. 2009). Using this same type of standard, Pierce et al. (2013) reported an LD of 6 molecules, slightly higher than our result, and with a dynamic range 2 orders lower.

Table 7. Summary of the limit of detection (LD) of viral haemorrhagic septicaemia virus (VHSV) and dynamic range. Ct: threshold cycle value; Order: order of magnitude of the dynamic range

	LD (no. of copies)	Dynamic range Ct	Dynamic range Copies	Order
$TCID_{50}$ ml^{-1}	1	14–36	~10^{-1} to 10^6	8
pDNA	2 (10 ag)	8–37	2 to 2.25×10^8	9
RNAtranscript	15 (10 ag)	11–37	15 to 1.5×10^8	8
Pure virus	16 (100 ag)	13–36	16 to 1.6×10^8	8
Crude virus	160 (1 fg)	14–35	160 to 1.6×10^8	7

The LD using an RNA transcript was raised to 15 copies, and with a dynamic range of 8 orders of magnitude: from 15 to 1.5×10^8 (Ct between 37 and 11). This result is similar to those reported for other viruses (Hoffmann et al. 2005, Workenhe et al. 2008), and features a much higher sensitivity than that reported by Garver et al. (2011) for the same virus (100 copies).

The use of a purified virus as the standard yielded the same LD as with a transcript (16 copies), which was expected because, to our understanding, the use of a transcript as a template is what best resembles the genomic RNA, and the purification of the virus ensures that only genomic RNA is available as a template. In terms of quantity of RNA, 100 ag is 10-fold lower than the LD observed with crude virus, and also 19-fold lower than that previously reported with a multiplex procedure (Cutrín et al. 2009).

These differences between the different standards have been widely described in the literature (Dopazo & Bandín 2011) and represent a handicap when results must be compared between studies. Therefore, the design of the present study was extended to ensure the reliability of the standard curves with each standard and chemistry, which would allow them to be compared. For that reason, the evaluation of the sensitivity with each type of reference was carried out with 3 replicates per day and 3 repetitions: on 3 different days by 3 different people. This design not only provided reliable LDs, but also allowed us to evaluate the repeatability and reproducibility of the procedure and, hence, the reliability of the final curve obtained for each standard and chemistry. As a result, we have demonstrated high levels of repeatability and reproducibility in all the experiments, regardless of the viral strain tested, as well as a high robustness, given that no significant effect of the experience of the worker was observed. In addition, all the standard curves showed high R^2 values and efficiencies within the range 90 to 110%, with all strains and standards, and regardless of the type of chemistry employed. All of these results support the high efficiency and reliability of this procedure for the diagnosis of VHSV isolates from any genotype.

The advantages and disadvantages of using real-time PCR with SYBR Green or with TaqMan probes are well documented (Dopazo & Bandín 2011). Consequently, SYBR Green RT-PCR is considered a flexible and cost-effective approach that can be directly applied without the need to design and synthesize fluorescently labeled target-specific probes (Martínez et al. 2008), and is less dependent on sequence variations. In addition, the post-amplification melting curve analysis provides highly specific detection (Kong et al. 2009), although further research is needed to ensure the specificity of the Tm. On the other hand, the advantage of using TaqMan chemistry for qRT-PCR lies in the highly improved specificity due to the use of a labelled internal probe. However, its disadvantage is the increased cost of the diagnosis with respect to the SYBR Green chemistry. Since our procedures have been demonstrated to provide standard curves statistically indistinguishable between both chemistries, the selection of either of them will only depend upon the preferences of each laboratory, ensuring the same reliability of diagnosis and quantification.

Data generated by real-time RT-PCR can be used to quantify native templates using either absolute or relative approaches. Absolute quantification requires the construction of a standard curve using relevant standards (Bustin 2000, Bustin et al. 2005, Wong & Medrano 2005); relative quantification describes the change in expression of the target gene relative to an internal normalised reference gene, usually a housekeeping gene (Giulietti et al. 2001). However, housekeeping genes are not necessarily appropriate references for qRT-PCR data normalisation except for estimating the quality of the RNA in a sample or for detecting the presence of inhibitory factors (Sellars et al. 2007). In the present study, we decided to choose an absolute quantification approach because to our understanding, it is a better approach for estimating the real number of copies of viral RNA in a sample. Nevertheless, we did include housekeeping genes (β-actin and 18S were tested) in the initial experiments (data not shown) to ensure that RNA extraction was correct. However, they were soon removed since they did not provide any special benefits or improve the procedure and, on the contrary, they made it more complicated and expensive. The repeatability and reproducibility of all of the results support our decision. In addition, other authors have reported the use of this same approach for quantification (Purcell et al. 2006, Bain et al. 2010).

The high repeatability and reproducibility also supports the use of the final standard curves as a reference for quantification, as long as the proposed protocol is followed as indicated. Given that no significant differences between the slopes of the standard curves of quantification were observed between the different references, which means that they can be considered parallel, a unique conversion factor between each pair of standards can be used to compare results from different studies that have used different templates as a reference for quantification,

and this will be very useful in the future, even to compare further studies with previous reports. Nevertheless, additional studies are being conducted to validate the procedures in infected fish samples.

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