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The use of endogenous and exogenous reference RNAs for qualitative and quantitative detection of PRRSV in porcine semen

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Abstract

Semen is known to be a route of porcine reproductive and respiratory syndrome virus (PRRSV) transmission. A method was developed for qualitative and quantitative detection of the seminal cell-associated PRRSV RNA in relation to endogenous and exogenous reference RNAs. As endogenous control for one-step real-time reverse transcription (RT)-PCR *UBE2D2* mRNA was selected. Particularly for the analysis of persistent infections associated with low copy numbers of PRRSV RNA, *UBE2D2* mRNA is an ideal control due to its low expression in seminal cells and its detection in all samples analysed ($n = 36$). However, the amount of *UBE2D2* mRNA in porcine semen varied (up to 106-fold), thus its use is limited to qualitative detection of PRRSV RNA. For quantitation, a synthetic, non-metazoan RNA was added to the RNA isolation reaction at an exact copy number. The photosynthesis gene ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) from *Arabidopsis thaliana* was used as an exogenous spike. Unexpectedly, PRRSV RNA was detected in a herd of specific pathogen-free (SPF) boars which were tested ELISA-negative for anti-PRRSV antibodies. Therefore, RT-PCR for seminal cell-associated PRRSV is a powerful tool for managing the SPF status during quarantine programs and for routine outbreak investigations.

Keywords: Internal positive controls; Exogenous and endogens reference RNAs; PRRSV; Real-time RT-PCR

1. Introduction

The porcine reproductive and respiratory syndrome virus (PRRSV) belonging to the *Arteriviridae* family is an enveloped single-stranded RNA virus with a plus-sense genome which replicates via a 3′-coterminal nested set of subgenomic mRNAs (Dea et al., 2000). The PRRSV-1 and PRRSV-2 strains (formerly European- and North American-type PRRSV, respectively; (Drew, 2000)) and the monophyletic Lithuanian PRRSV strains (Plagemann, 2003; Stadejek et al., 2002) can be differentiated. In semen, PRRSV may be shed from the bulbourethral gland (Christopher-Hennings et al., 1995) and can be located in immature sperm cells (Sur et al., 1997) or macrophages (Christopher-Hennings et al., 1998). PRRSV can be detected in semen as early as 2–3 days postinoculation (p.i.) (Christopher-Hennings et al., 1995; Legeay et al., 1997) and can be transmitted by insemination (refs. in Sur et al., 1997). PRRSV-infected boars show no significant clinical signs, and seroconversion and/or viremia is not correlated with shedding of virus in semen (Wills et al., 2003). While another arterivirus, equine arteri-

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tis virus, is shed in semen for at least 1–2 years, a prolonged carrier state (>day 101 p.i.) in PRRSV-infected boars has not been reported (Christopher-Hennings et al., 1995).

Reference RNAs (internal positive controls, internal control RNAs) can be classified into exogenous (spike-in RNA, RNA extraction control, exogenous reference transcript) and endogenous controls. Exogenous controls spiked at a defined copy number to the sample before RNA isolation and endogenous controls can be applied for relative quantitation of viral RNAs using real-time RT-PCR. Therefore, reference RNA expression should be comparable with that of the target and independent from the experimental conditions, e.g. the presence of a virus. Both reference types serve as controls for sample transport and storage conditions, isolation performance, normalise for differences in total RNA input or contribute to the detection of false negative results caused by sample-specific inhibitors. They were applied for monitoring the course of infection or intermittent shedding of viruses and for differentiation between a viremic or persistent stage of infection (Moody et al., 2000; Pasquier et al., 2003). Exogenous controls are used for testing of cell-free body fluids. Their copy number can be controlled precisely and adjusted easily to the copy number of the gene of interest. Endogenous controls are a cost efficient alternative for the detection of viral RNAs in veterinary diagnostic work.

Artificial insemination or conventional sexual reproduction-mediated PRRS virus transmission is of great importance for PRRSV epidemic. For detection of PRRSV in boar semen candidate endogenous reference genes were selected among common housekeeping genes and other genes found previously to be expressed uniformely in human and mouse tissues (Hamalainen et al., 2001; Warrington et al., 2000). They were chosen from different functional categories based on the Panther classification system (Thomas et al., 2003) which reduces significantly the chance that genes might be co-regulated (Vandesompele et al., 2002). For the development of one-step real-time RT-PCR for four endogenous reference RNAs (*HPRT*, *UBE2D2*, *PPIA*, and *HMBS*) appropriate target regions were selected and the assay conditions were optimised for amplification efficiency. Next, their expression levels and the amplification from genomic DNA contamination were examined. The *UBE2D2* mRNA was found to be a suitable endogenous control for qualitative molecular diagnostics of PRRSV RNA in the cell-associated part of porcine semen. However, due to the variable expression of *UBE2D2* mRNA, an exogenous reference RNA derived from a plant gene was developed for quantitative detection of the viral RNA.

2. Materials and methods

2.1. Biological material

The PRRSV-1 reference sample used for assay validation was generated as follows. A 9-week-old piglet of a cross-bred (Landrace × Large White) tested negative for antibodies against PRRSV was infected artificially with a $10^{6.5}$ $TCID_{50}$ of the Spanish isolate Olot/91 (PRRSV-1, GenBank accession number X92942) and slaughtered at day 28 p.i. Lung tissue was shipped on dry ice and kept at −80 °C until RNA isolation.

Lung tissues (22 PRRS viral RNA-negative and 23 PRRSV-1- or/and PRRSV-2-positive) for studying *UBE2D2* mRNA expression originated from the breeds Large White, Landrace, Piétrain, Large White × Piétrain and (Large White × Landrace) × Piétrain.

Ejaculates were derived from two boar herds (set A and B boars). The set A boars ($n = 12$; Piétrain, Landrace, and Large White breed) were 1–5.5 years old and lived in the herd since 6–57 months. The boars of set B ($n = 9$; Piétrain) which joined the herd at the age of 8 months were between 20 and 57 months old. Both sets of boars were housed under specific pathogen-free conditions. This was guaranteed by the quarantine and testing program for incoming boars involving two serological tests with an interval of 2 weeks against classical swine fever virus (CSFV), pseudorabies virus (PRV), swine influenza virus (SIV), transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), *Chlamydophila*, *Brucella*, and *Leptospira* species. After quarantine, the boars are tested for these pathogens once a year. According to the management program for the set A boars the absence of PRRSV antibodies was required. This was achieved by testing routinely with the IDEXX (HerdChek) ELISA (IDEXX Laboratories, Woerrstadt, Germany) for which a sample-to-positive (s/p) ratio of ≥0.4 is considered positive. At the time of sampling the set A boars were ELISA negative (−0.08 to 0.18). In addition an immunoperoxidase monolayer assay (IPMA (Wensvoort et al., 1991)) confirmed the PRRS-negative serology. The set B boars showed a high positive serostatus when semen samples were taken (IDEXX ELISA values: 2.4–2.7 for $n = 5$, and 1.0–1.6 for $n = 4$) indicating a recent contact with PRRSV.

2.2. RNA extraction

Total RNA from tissue samples and viral RNA contained in the Porcilis® PRRS vaccine (Intervet, Unterschleissheim, Germany) were isolated with the TRIzol® Reagent and the TRIzol® LS Reagent (Invitrogen, Lofer, Austria) according to the instructions of the manufacturer and finally dissolved in 50 μl diethyl pyrocarbonate (DEPC)-treated water. The QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA from 140 μl serum samples and from the modified live PRRSV vaccine (Ingelvac® PRRS MLV; Boehringer Ingelheim Pharma, Ingelheim am Rhein, Germany) applying 60 μl elution buffer. The vaccine is a cell culture-adapted derivative of the pathogenic PRRSV-2 isolate deposited at the American Type Culture Collection under the number ATCC-VR2332 (GenBank: U87392).

For seminal RNA isolation ejaculated boar semen was collected into a sterile container with an integrated filter

(US BAG™, Minitube, Tiefenbach bei Landshut, Germany) and was immediately stored at 4 °C to guarantee that the non-sperm cells remained intact. The interval until further processing did not exceed eight hours. RNA was isolated using the GenElute™ Mammalian Total RNA Kit (Sigma-Aldrich, Vienna, Austria) with modifications. From a 30 μl-semen aliquot the cellular fraction was pelleted by centrifugation at 3000 × *g* for 4 min. The pellet was lysed by vortexing for 5 min in 500 μl lysis buffer containing 143 mM 2-mercaptoethanol. The RNA was eluted in a volume of 50 μl and stored at −80 °C.

2.3. Plasmid construction and preparation of PRRSV-2 ORF6 and rbcL RNA standards

The plasmid p416BSK (M.P. Murtaugh, University of Minnesota, USA) containing a 2 kb fragment of the PRRSV-2 reference strain VR2332 and an *Arabidopsis thaliana* cDNA prepared as reported previously (Karsai et al., 2002) were used as PCR templates. Amplification was carried out with the primers clUS-ORF5-f3 and clUS-ORF7-r5, and rbcL1307f and rbcL1584r (*rbcL* gene; GenBank: ATU91966), respectively. PCR products were gel purified and subcloned into the pGEM®-T Easy Vector System I (Promega, Mannheim, Germany). Plasmids were purified by a modified alkaline lysis method, verified by DNA sequencing, linearised by *Sal*I digestion and recovered by phenol-chloroform extraction followed by ethanol precipitation. In vitro transcription reactions were performed in 20 μl containing 1 μg linearised plasmid, 1× transcription buffer with 10 mM DTT, 0.5 mM of each rNTP and 20 U T7 RNA Polymerase (MBI Fermentas, St. Leon-Rot, Germany) for 1 h at 37 °C. Subsequently, the reaction was incubated with 1 U RNase-free DNase I (Ambion, Austin, USA) at 37 °C for 15 min. In vitro transcripts were recovered by phenol-chloroform extraction and ethanol precipitation and quantitated by spectrophotometry.

2.4. Real-time RT-PCR assay design for endogenous reference RNAs

The housekeeping genes *HPRT* (GenBank: AF143818), *UBE2D2* (*UBCH5B*), *PPIA* (*CYPA*; GenBank: AY008846), and *HMBS* (*PBGD*) were selected as candidate endogenous reference mRNAs considering previous reports (Foss et al., 1998; Hamalainen et al., 2001; Steele et al., 2002) and the fact that in contrast to *HPRT*, *UBE2D2* and *PPIA* there is no pseudogene for *HMBS* in the human genome ((Zhang et al., 2004); www.pseudogene.org). Human PCR primer sequences targeting conserved regions were selected in a human/mouse-nucleotide alignment (GenBank: U39317 and M95623) to determine partial porcine *UBE2D2* and *HMBS* cDNA sequences and subsequently to select primers and probes for real-time RT-PCR of *UBE2D2* and *HMBS*. In addition to PCR product sequencing the partial porcine *HMBS* cDNA was determined by sequence analysis of subclones derived from tissue cDNAs (data not shown).

2.5. Sequencing

The amplification of cDNA for PCR product sequencing was carried out in a 25 μl-reaction volume containing 2 μl of the reverse transcription reaction, 1× buffer, 2 mM magnesium, 0.2 mM of each dNTP, 300 nM of each primer and 1 U *Taq* DNA polymerase. Following denaturation at 95 °C for 4 min, targets were amplified by 35 cycles at 95 °C for 20 s, 59 °C for 30 s and 72 °C for 1 min. For sequencing the ABI PRISM BigDye™ Terminator Cycle Sequencing chemistry and the ABI PRISM 377 sequencer (Applied Biosystems, Vienna, Austria) were used.

2.6. One-step real-time RT-PCR

One-step quantitative real-time RT-PCR was carried out with a two-enzyme system. Separate reverse transcription and DNA polymerisation reactions allow testing for DNA contamination by including a “minus RT” control, thus assaying for amplification from processed and non-processed pseudogenes. The two-enzyme system used (TaqMan® One-Step RT-PCR Master Mix Reagents Kit, Applied Biosystems) contains the Moloney Murine Leukemia Virus Reverse Transcriptase and AmpliTaq Gold® DNA Polymerase. A volume of 5 μl RNA per 25 μl reaction and primer/probe concentrations of 600 nM/250 nM and 300 nM/200 nM were applied for the viral and the reference RNA assays, respectively. Total RNA was reverse transcribed for 30 min at 48 °C. The hot start DNA polymerase was activated by incubation at 95 °C for 10 min followed by amplification for 45 cycles at 95 °C for 15 s and 60 °C for 1 min.

Fluorescence “real-time” measurements recorded by either the ABI PRISM® 7700 or 7900HT Sequence Detection Systems (Applied Biosystems) were transformed into C_T values using the SDS software versions 1.9.1 and 2.1, respectively.

2.7. Generation of standard curves

Dilution series of standard RNAs were made to characterise the linearity, precision, specificity, and sensitivity of the quantitation assays. Standard dilutions were prepared in DEPC-treated water containing 30 ng/μl yeast tRNA carrier (Invitrogen) and were amplified at least in duplicates. The C_T values were plotted either against the log of the target copy number or against the log of the input RNA mass to generate a regression curve with the formula $y = sx + b$ (s, slope; b, intercept) and to determine the reaction efficiency $E = 10^{-1/s} - 1$. As internal quality parameter for a standard curve a regression coefficient (R^2) of >0.990 was used. Optimal efficiency was concluded if $E \geq 0.9$ ($s < -3.6$).

2.8. Selection of primers and probes for PRRSV detection

To identify conserved sites for primer and probe binding in the *ORF6* gene coding for the most conserved structural protein (M proteins of PRRSV-1 and -2 show amino acid sequence identity of 78–81% (Dea et al., 2000)) the following sequences were aligned using the Sequence Navigator software (Applied Biosystems): (i) seven PRRSV-1 sequences: M96262, AJ223078, X92942, L04493, AF298882, AF511525, AF512378, and (ii) 44 PRRSV-2 sequences: U87392, AF066183, AF066384, AJ223082, L40898, U75443, AF188680, AF066068, U64928–U64935, Z82995, AF046869, L39361-L39369, U03040, U18748–U18752, AB023782, D45852, AF035409, AF121131, AF030306, AF090173, AF184212, AF132118, and AJ223079-AJ223081.

2.9. Primers and probes

Primers (Invitrogen), TaqMan probes (MWG Biotech, Ebersberg, Germany) and TaqMan Minor Groove Binder (MGB) probes (Applied Biosystems, Weiterstadt, Germany) were designed using the Primer Express™ 1.5 software (Applied Biosystems, Vienna, Austria) and are given below as 5′–3′ sequence. Standard TaqMan probes were labeled with the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) and with the reporter dyes 6-carboxyfluorescein (6FAM) or 6-carboxy-4,7,2′,7′-tetra-chlorofluorescein (TET). In the TaqMan MGB probe format a non-fluorescent quencher dye (NFQ) and a minor groove binder (Kutyavin et al., 2000) were applied.

The complete *ORF6* and the partial *ORF7* sequences were determined by PCR product sequencing using the primer pairs EU5-546f (CCTCGAAGGGGTTAAAGCTCA) and EU6-462r (CACGAGGCTCCGAAGTCCT), and EU6-343f (GTAGAAAGTGCTGCAGGTCTCCA) and EU7-340r (GCACTGTATGAGCAACCGG), respectively. In addition, the primers clEU5-562f (GCTCAACCCTTGACGAGGACT), clEU7-10r (GCTCTGGTTTTTACCGGCC), clUSORF5-f3 (ATAACCAGAGTTTCAGCGGAACA), clUSORF7-r5 (TCTCTTCTGCTGCTTGCCGT), rbcL1307f (CTTGAAGGAGACAGGGAGTCAACT) and rbcL1584r (CATGCTTCCAGAGCTACTCGG) were applied. To determine the partial porcine sequences for the *HMBS* housekeeping version and for *UBE2D2*, the primers HsHMBS-f (ATGTCTGGTAACGGC, exon 1), HsHMBS-r (GGGTACGAGGCTTTC, exon 4) and hUBE2D2-seq8f (TGAAGAGAATCCACAAGGAATTGA, exon 2) and hUBE2D2-seq375r (CCGAGCAATCTCAGGCACTAAA, exon 7) which were deduced from the homologous human genes (GenBank: NM_000190 and U39317) were used. Primers and probes for the endogenous control assays for *PPIA* and *HPRT* developed on the basis of known porcine sequences (GenBank: AY008846 and AF143818) were pCYC-30f (GCCGCGTCTCCTTCGAG), pCYC-133r (GCAGGAACCTTTATAACCAAATCCT) and pCYC-VIC/MGB (VIC-CAGAAAACTTCCGTGCTC-NQF-MGB), and pHPRT-114f (TCATGGACTAATTATGGACAGGACTG), pHPRT-344r (TTTATATCGCCCGTTGACTGGT) and pHPRT-TET/TAMRA (TET-TGGGAGGCCATCACATCGTAGCC-TAMRA). Oligonucleotides for quantitation of porcine *HMBS* and *UBE2D2* mRNAs based on sequences determined in this study were pHMBS-24f (CGCAACGGCGGAAGAAA), pHMBS-128r (TTCAGCGTTGCCACCACAC) and pHMBS-84FAM/TAMRA (6FAM-CCAGCTGGCCCGCATACAAACG-TAMRA); pUBE2D2-70f (AGGTCCTGTTGGAGATGATATGTT), pUBE2D2-173r (TTGGGAAATGAATTGTCAAGAAA) and pUBE-2D2-118FAM/TAMRA (6FAM-CCAAATGACAGTCCC-TATCAGGGTGGA-TAMRA).

Real-time RT-PCR for PRRSV-1 targeted the *ORF6* gene with the primers EU6-343f and EU6-462r (see above) and the TaqMan MGB probe EU6-MGB (6FAM-CTGTGAGAAAGCCCGGAC-NFQ-MGB). The *ORF6* sequence of PRRSV-2 was quantified with the primers US6-289f (TCCAGATGCCGTTTGTGCTT) US6-412r (GACGCCGGACGACAAATG) and the probe US6-MGB (6FAM-CCCTGCCCACCACGT-NFQ-MGB). Both probe binding sites were completely homologous among the respective PRRSV sequences listed above.

The primers rbcL1307f (see above) and rbcL1388r (TACCGCGGCTTCGATCTT) were applied in combination with the TaqMan probe rbcL1356TET (TET-TCGCGCAGTAAATCAACAAAGCCCA-TAMRA) to quantitate the exogenous RNA standard.

If possible primer and probe sequences for real-time PCR were selected to target regions with no obvious secondary structure using the RNA and DNA folding server (www.bioinfo.rpi.edu/~zukerm/).

A significant amplification from non-processed and processed pseudogenes of endogenous reference genes during real-time RT-PCR was prevented by primer annealing across an exon boundary (*UBE2D2* and *HPRT* assays) and by primers flanking a large intron (*HMBS* and *PPIA* assays). The porcine exon-intron structure was concluded from the homologous human and mouse genes (Batzoglou et al., 2000). We also considered that the human and the mouse genomes contain a processed pseudogene for *UBE2D2*, but not for *HMBS* (www.pseudogene.org).

2.10. Statistics

The qualitative detection of the endogenous reference *UBE2D2* mRNA in PRRSV-infected and non-infected pigs was evaluated by the Student's *t*-test and by the non-parametric Wilcoxon Rank-Sum (Mann-Whitney) test for two independent samples included in the statistical analysis software SPSS for Windows 9.0.1 (SPSS Inc., Chicago, USA). The Spearman's rank correlation test was used to analyse the relationship between *UBE2D2* expression in semen RNAs and the sperm cell concentration.

2.11. Assay identification and sequence accession numbers

The (porcine) *UBE2D2* real-time RT-PCR assay was submitted to the Real-Time PCR Primer and Probe Database (RTPrimerDB ID: 1240; (Pattyn et al., 2003)) and can be used for detection of human *UBE2D2* mRNA due to complete primer/probe homology.

Sequences determined within this study were submitted to the GenBank under the following accession numbers: AF511525 (Olot/91-G2540A), AF511526 (Porcilis PRRS vaccine), AY1482221 (*HMBS*), AY148222 (*UBE2D2*), AF143818 (*HPRT* variant) and AF543683 (*PPIA*-like pseudogene). The sequence of the Austrian PRRSV-1 isolate AGS-96 (AF512378) which mismatched in the center of the *ORF7* probe binding site targeted by a previous TaqMan RT-PCR assay (Egli et al., 2001) was also submitted.

Endogenous control genes were designated according to the homologous human genes using LocusLink (www.ncbi.nlm.nih.gov).

3. Results

3.1. Reference mRNAs for detection of PRRSV RNA in the cellular fraction of semen

The dynamic ranges and the amplication efficiencies of the real-time RT-PCR assays for *HPRT*, *UBE2D2*, *PPIA*, and *HMBS* were analysed by generating standard curves using lung and lymphoid tissue RNAs (Fig. 1a, and data not shown). The equations for the regression lines illustrate that the reaction efficiencies for the endogenous control assays ($E > 0.9$, i.e. optimal) were comparable to those of the viral assays ($\Delta E < 10\%$). A DNase I treatment step to exclude cross-amplification from genomic DNA was made redundant by assay design/selection (see Section 2). Using porcine tissue RNAs we demonstrated that amplification from any contaminating DNA was not significant (ΔC_T for mRNA and the corresponding minus RT control > 6).

For RNA quantitation in semen specimens *UBE2D2* and *HMBS* were analysed in more detail since in semen the differences between the C_T value for *PPIA* or *HPRT* and the corresponding minus RT sample controling for genomic DNA contamination were small (data not shown). The *UBE2D2* and *HMBS* expression was analysed in native semen samples derived from two sets of boars (A, B) with negative and high positive PRRSV serostatus. Independently of the PRRSV serostatus or presence of low PRRSV RNA copy numbers (see below), the C_T values obtained for *HMBS* mRNA were at or near the detection limit (Table 1; semen RNAs for set B boars were not analysed), whereas the *UBE2D2* mRNA was detected in all 36 ejaculates of the 22 individuals analysed (Table 1 and data not shown). No correlation between *UBE2D2* expression and the sperm cell number was found (Spearman Correlation Coefficient $r = -0.057$, $p = 0.81$; $n = 20$ in Table 1). The *UBE2D2* expression varied from 5.3-fold (set B boars, $n = 4$, $C_T > 45$ for PRRSV RNA, positive PRRSV serostatus), over 45-fold (set A boars, $n = 12$, seronegative for PRRSV) to 106-fold (set B boars, $n = 5$, only one semen RNA with detectable PRRSV-1 *ORF6* copy numbers, $C_T > 45$ for PRRSV-2 RNA, high positive PRRSV serostatus).

To prove that *UBE2D2* would be invariant in the primer and probe binding sites among different individuals of common pig breeds random porcine cDNAs ($n = 9$) were sequenced. Complete homology in the binding sites was found. Moreover, human and pig *UBE2D2* nucleotide sequences were completely homologous in the primer/probe binding sites.

Since in semen the variation of *UBE2D2* expression exceeded the high stringency criteria applied in gene expression studies (variation < five-fold (Dheda et al., 2004)), we developed a non-metazoan exogenous control RNA spike. This reference RNA is based on the chloroplast-encoded gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) from *A. thaliana* (Clegg, 1993). The standard curve obtained for the in vitro transcript documents optimal amplification efficiency (Fig. 1b). Spiking the cellular fraction of a semen RNA sample with 37,500 copies of the *rbcL* in vitro transcript, C_T values of 30.9 and 30.3 were obtained.

3.2. RNA isolation protocol for the quantitation of PRRSV in semen specimens

A silica membrane-based kit was selected to standardise isolation for quantitation of PRRSV RNA in semen specimens. Different semen volumes and different sperm cell numbers were tested with a constant amount of absorbent material (2 mm silica column) to determine the lowest C_T value for a target mRNA (*UBE2D2* mRNA) by one-step real-time RT-PCR. Cell numbers of the twelve experimental ejaculates ranged from 1.28 to 4.81×10^8 cells/ml. Duplicate C_T measurements for the 10, 20, 30 and 40 μl semen aliquots ranged between 31.3 ± 0.1 and $C_T > 45$ (no amplification). The lowest C_T values were obtained for the 30 μl and/or the 40 μl semen aliquots (C_Ts: 31.3 ± 0.1 to 36.7 ± 0.2, and 32.0 ± 0.5 to $C_T > 45$). No correlation was observed between C_T value and the number of spermatozoa. The successful removal of inhibitors for real-time RT-PCR was obtained since one-step real-time RT-PCR yielded optimal amplification efficiency ($E > 0.9$ for *UBE2D2* mRNA, PRRSV-1 and -2 RNAs; Fig. 1a).

3.3. Quantitation assays for PRRSV-1 and -2 RNAs

One-step real-time RT-PCR assays for PRRSV-1 and -2 RNA allowed quantitation with optimal efficiency (Fig. 1a; standard curves for two additional viremic pigs infected with PRRSV-1 (data not shown)) as achieved for endogenous and

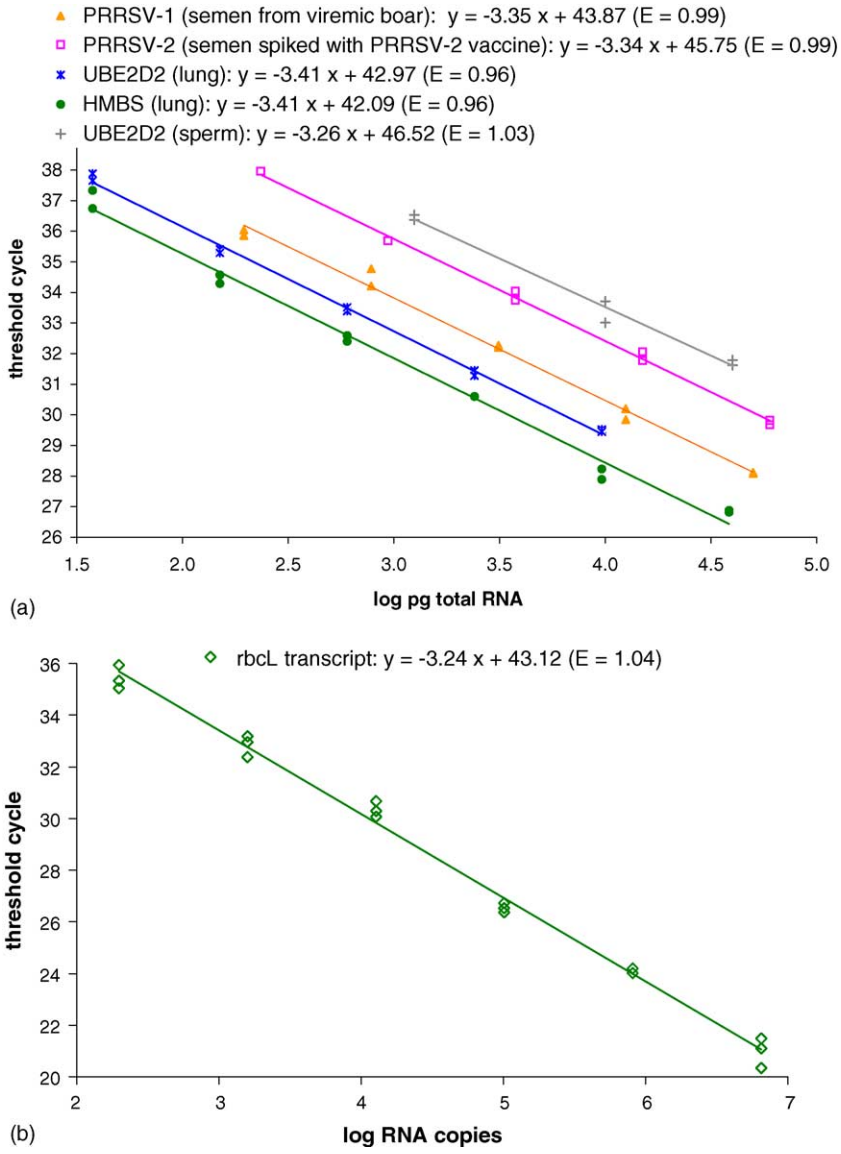

Fig. 1. One-step real-time RT-PCR yields optimal amplification efficiency for quantitation of PRRSV RNAs, endogenous (a) and exogenous reference RNAs (b). $C_T > 45$ for minus RT controls of *UBE2D2* and *HMBS* mRNAs. PRRSV-2 infection was mimicked by spiking virus-free semen with the Ingelvac® PRRS MLV vaccine.

exogenous reference RNA assays ($\Delta E < 10\%$, Fig. 1 and see above).

Since a variation in the detection limits for the different PRRSV strains due to SNPs within the amplicon (Barnard et al., 1998) or in the primer binding sites cannot be excluded, the sensitivity of the assay for a single PRRSV reference strain (ATCC-VR2332, PRRSV-2) was determined. For this purpose, PRRSV RNA-free seminal cell fraction-derived RNA (see below) was spiked with known copy numbers of the PRRSV-2 *ORF6* in vitro transcript (serial four-fold dilutions). The resulting detection limit for the analysis of PRRSV-2 RNA in the cellular fraction of semen was 20 *ORF6* RNA copies.

The specificity of the real-time RT-PCR assays for PRRSV-1 and -2 was examined by using viral RNA from the opposite PRRSV type as a non-amplification control. Viral RNA contained in the Porcilis PRRS vaccine (PRRSV-1) and the Ingelvac PRRS MLV vaccine (PRRSV-2) was purified and used at a concentration of 2.4×10^6 *ORF6* copies/reaction as target and non-target copy numbers (and vice versa) in the quantitation assays. These copy numbers exceeded several-fold the copy numbers found in clinical samples of serum and sperm (data not shown). The real-time RT-PCR quantitation assays ($E > 0.9$) did not detect any non-target amplification demonstrating absence of cross-amplification between the two PRRSV types (data not shown). The absence of any cross-amplification with samples tested positive for classical swine fever virus (CSFV), transmissible gastroenteritis virus (TGEV), porcine parvovirus (PPV), pseudorabies

Table 1
UBE2D2 mRNA as endogenous control for qualitative detection of PRRSV RNA

Boar number (set)	C_{T1}/C_{T2} for *HMBS* mRNA	C_{T1}/C_{T2} for *UBE2D2* mRNA	IDEXX ELISA[a]	C_T for PRRSV-1 in semen[b]	C_T for PRRSV-1 in serum[b]	Cell number × 10^8/mL	Breed
1 (A)	>45/>45	33.3/33.6	0.03	>45	>45	4.81	P
2 (A)	>45/>45	35.0/34.8	0.05	>45[c]	>45	3.82	L
3 (A)	35.6/33.9	31.1/31.3	−0.02	>45[c]	>45	2.37	LW
4 (A)	36.6/>45	36.3/37.1	0.06	>45	>45	2.11	P
5 (A)	36.7/36.7	36.3/35.5	0.18	>45[c]	>45	3.29	L
6 (A)	36.6/35.9	35.3/36.2	−0.01	>45[c]	>45	2.22	P
7 (A)	>45/34.8	34.3/34.4	0.05	>45[c]	35.0	1.28	P
8 (A)	34.2/33.4	31.7/32.7	−0.01	>45	34.9	3.14	P
9 (A)	30.5/30.3	30.6/32.0	−0.07	>45[c]	>45	4.00	L
10 (A)	33.5/33.4	33.2/34.0	0.15	>45[c]	>45	2.45	P
11 (A)	>45/34.5	34.8/35.6	0.04	>45[c]	34.6	2.91	L
12 (A)	33.3/32.9	34.9/35.5	−0.08	34.6	35.6	2.82	P
13 (B)	n.a.	31.5/31.9	2.36	>45	n.a.	n.a.	P
14 (B)	n.a.	37.1/36.7	2.56	>45	n.a.	5.73	P
15 (B)	n.a.	35.7/35.8	2.64	34.7	n.a.	1.35	P
16 (B)	n.a.	34.3/35.1	2.65	>45	n.a.	2.43	P
17 (B)	n.a.	38.0/38.8	2.59	>45	n.a.	2.47	P
18 (B)	n.a.	33.7[d]	0.95	>45	n.a.	2.92	P
19 (B)	n.a.	36.1[d]	1.03	>45	n.a.	5.86	P
20 (B)	n.a.	35.3[d]	1.37	>45	n.a.	2.02	P
21 (B)	n.a.	34.3[d]	1.60	>45	n.a.	3.17	P

P, Piétrain; L, Landrace; LW, Large White; n.a., not analysed; $C_T > 45$, undetectable signal.
[a] Negative if s/p ratio ≤ 0.4.
[b] $C_T > 45$ for PRRSV-2.
[c] *ORF6* RNA of PRRSV-1 was detected 69 days before.
[d] Unicate amplification.

virus (PRV) and porcine circovirus type II (PCV2) was also confirmed (data not shown). Using the amplicon sequences of the PRRSV real-time RT-PCR assays as query in a nucleotide–nucleotide BLAST (blastn) the specificity of the PRRSV primer and probe sequences was demonstrated.

The PRRSV-1 and -2 quantitation assays were used for examination of semen samples derived from two sets of boars with negative (A) and high positive (B) PRRSV serostatus (see Section 2). Unexpectedly, semen samples with PRRSV-1 RNA copy numbers near the detection limit were detected in both sets (Table 1 and data not shown). Four of the twelve sera of the set A boars were positive for PRRSV-1 RNA but negative for PRRSV-2 RNA (Table 1; serum RNAs for set B boars were not analysed).

4. Discussion

A method was developed for qualitative and quantitative detection of the seminal cell-associated PRRSV RNA in relation to endogenous (*UBE2D2* mRNA) and exogenous (*rbcL* RNA) reference RNAs. Neither rRNA expression or total RNA amount (reviewed by Bustin, 2002) are applicable for normalisation of relative quantitation of PRRS viral RNA in the seminal cell fraction due to the large difference in expression between viral and rRNA genes during persistent infections and the limited practicability of spectrophotometric analysis for routine diagnostic application.

UBE2D2 mRNA is an appropriate control for routine qualitative detection of PRRSV RNA due to its reliable and low expression in all semen samples tested. Preliminary data indicate similar expression of *UBE2D2* mRNA in animals with PRRS viral RNA-positive or negative status (C_Ts for tissue samples: 29.6 ± 3.2 and 30.7 ± 3.6, respectively). However, we cannot exclude that for example a selection of the individuals in the two groups based on age, breed, stage of infection or a study design testing in the same animal under the same conditions with and without PRRSV would reveal differential expression of *UBE2D2* depending from the status of PRRSV infection. The C_T values determined for *UBE2D2* mRNA in semen were comparable to those determined for samples with low viral RNA copy numbers which is especially important for reliable virus quantitation during persistent infections. The reasons for the varying C_T values in the *UBE2D2* assay ($\Delta C_T = 7.2$, Table 1) were not addressed and might involve differences in the RNA quality among the samples, in the number and/or the expression level of *UBE2D2* mRNA expressing cells. Summarising, the validation of an endogenous reference which would be a cost-efficient alternative for quantitative detection of PRRSV still remains a challenge due to the heterogeneous cell population of semen and its varying cell number.

The real-time RT-PCR assays for *HPRT*, *PPIA*, *HMBS* and *UBE2D2* developed in this study will be useful for future gene expression studies in the pig.

Due to the occurrence of processed pseudogenes (or intron-less paralogs), housekeeping gene mRNAs used in a

previous study as endogenous references for human semen samples required the application of DNase I (Juusola and Ballantyne, 2003). This pretreatment step is assumed to impair RNA quality and increases assay-related costs and time. Here we show that it is possible by a careful primer and probe design to prevent significant amplification from genomic DNA without a DNase I digest.

Quantitation of viral RNA relative to a reference RNA requires knowledge of the exact copy number contained in this control. Due to the considerable variation (or complete absence in the case of *HMBS* mRNA in two samples) in expression in porcine semen RNA among the candidate reference genes studied we replaced the endogenous control by an exogenous control RNA. It was selected to avoid cross-hybridisation with RNAs from the organism studied. In the present study the photosynthesis gene *rbcL* from *A. thaliana* (Clegg, 1993) was used. Artificial sequences or sequences from other organisms are also possible as exogenous controls (Baker and O'Shaughnessy, 2001; Smith et al., 2003). In contrast to a homologous exogenous RNA (mimic) which has the same primer but different probe binding sites as the target RNA (Westcott et al., 2003), the use of a heterologous exogenous reference can be applied easily for detection of different target RNAs (for review see Freeman et al., 1999).

Real-time RT-PCR assays for detection of viral RNA in the plasma fraction of semen have been reported (Balasuriya et al., 2002; Westcott et al., 2003). PRRSV appears most consistently in the cellular fraction of semen and not in whole semen or seminal plasma (Christopher-Hennings et al., 1995, 1998). We therefore developed a quantitation assay for seminal-cell associated PRRSV RNA removing the seminal plasma which has been noted to contain PCR inhibitors (refs. in Bourlet et al., 2003).

The real-time RT-PCR assays for the endogenous and exogenous references as well as for PRRSV-1 and -2 yielded optimal efficiency ($E = 0.96$ to 1.04). This allows the relative quantitation of viral RNA by the $\Delta\Delta C_T$-method (ref. in Pfaffl, 2001) avoiding the elaborate amplification of standards in parallel.

The detection limit of the PRRSV real-time RT-PCR was determined for PRRSV-2 only. Future assay validation should involve (i) sequence analysis of the assay target region for new isolates, (ii) the use of primers and/or probes which are degenerated at defined nucleotide positions in order to maximise the detection of all known strains and isolates, (iii) determination of efficiency and sensitivity for isolates varying in the primer and/or probe target sites, and (iv) spiking of quantitation standards which represent these polymorphic isolates into an adequate number of semen samples before RNA extraction.

The occurrence of viral RNA in serum and semen (set A boars in Table 1) of seronegative boars housed under SPF conditions was unexpectedly demonstrated. The high C_T values reflect low copy numbers, thus virus isolation would not be expected to be successful (Wills et al., 2003). The occurrence of seroconversion is highly unlikely due to these low C_Ts, considering the routine PRRS antibody testing carried out in the SPF boar stud and the fact that eight of these boars were ELISA- and IPMA-negative 69 days after showing low C_Ts for PRRS viral RNA in semen (superscript b in Table 1).

The sporadic detection of viral RNA in serum after a period of negative testing was reported, and pigs which have returned to seronegative status based on ELISA may still harbor infectious PRRSV (Batista et al., 2004; Martelli et al., 2004; Wills et al., 2003). The serological status is also not an adequate indicator of PRRSV and PRRSV RNA shedding in the semen (Christopher-Hennings et al., 1995). This underlines the need to complement immunoassays with more sensitive nucleic acid-based methods for a comprehensive approach especially for the characterisation of persistent PRRSV infections (Batista et al., 2004), e.g. during the quarantine and testing program for incoming boars to reduce later conflicts in the interpretation of the PRRSV status. It is possible that our results obtained for the boars of set A, retrospectively, document only a contact between the animals and the virus before joining the herd, since the incoming boars were selected on the basis of their serological, but not of their viral RNA status, and contact of the boars with PRRSV before entering the herd was possible in principle.

Note

During the processing of this paper, quantitative real-time RT-PCR tests for the detection of PRRSV in whole boar semen and in the cell-associated part of the ejaculate were reported, respectively (van Rijn, P.A., Wellenberg, G.J., Hakze-van der Honing, R., Jacobs, L., Moonen, P.L., Feitsma, H., 2004. Detection of economically important viruses in boar semen by quantitative RealTime PCR technology. J. Virol. Meth. 120, 151–160; Wasilk, A., Callahan, J.D., Christopher-Hennings, J., Gay, T.A., Fang, Y., Dammen, M., Reos, M.E., Torremorell, M., Polson, D., Mellencamp, M., Nelson, E., Nelson, W.M., 2004. Detection of U.S., Lelystad, and European-like porcine reproductive and respiratory syndrome viruses and relative quantitation in boar semen and serum samples by real-time PCR. J. Clin. Microbiol. 42, 4453–4461).

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Competing interests statement

While S.R.-F. and G.B. declare their commercial interest in PRRSV molecular diagnostics, the remaining authors have no competing financial interests.

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