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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 endogenous 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; Studsjek 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-
However, due to the variable expression of endogenous control for qualitative molecular diagnostics of 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 uniformly 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 HMB5) 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 10³ TCID₅₀ 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), Chlamydomphila, 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 (Herd-Chek) ELISA (IDEXX Laboratories, Woerststadt, 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™, Miniture, 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 in vitro transcription reactions were performed in 20 μl containing 1 μg linearised plasmid, 1 μl 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 10 mM DTT, 0.5 mM of each rNTP and 20 U T7 RNA 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.

2.5. 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 primer/probe concentrations of 600 nM/250 nM and 1× reaction 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 quantified by spectrophotometry.

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

The housekeeping genes HPRT (GenBank: AF143818), UBE2D2 (UBCH5B), PPIA (CYP4A; GenBank: AY008846), and HMBS (PGD) 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).
2.8. 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, AF223078, X92942, L40896, AF298882, AF511525, AF512378, and (ii) 44 seven PRRSV-1 sequences: M96262, AJ223078, X92942, L40896, AF298882, AF511525, AF512378, and (iii) 44 PRRSV-2 sequences: U87392, AF066183, AF066384, L04493, AF298882, AF511525, AF512378, and (iv) 44 PRRSV-2 sequences: U87392, AF066183, AF066384, L04493, AF298882, AF511525, AF512378.

The ORF6 sequence of PRRSV-2 was quantified with the primers US6-289 (CGGCCGGAGCACAAAGT) 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 (TACCCGCGCTTGGACTC) were applied in combination with the TaqMan probe rbcL1356TET (TET-TGGGAGGCCATCACATCTCAGC-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 PPA assays). The Porcine exon-intron structure was concluded from the homologous human and mouse genes (Batzoglou et al., 2000). 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.

2.9. 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 (NFQ) and a reporter dye 6-carboxyfluorescein (6FAM) or 6-carboxytetra-chlorofluorescein (TET). In the TaqMan MGB probe format a non-fluorescent quencher dye (NFQ) and a fluorescent quencher dye (NFQ) and a fluorescent quencher dye (NFQ)

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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 (RT PrimerDB: 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 GenBank under the following accession numbers: AF511525 (Olot/91-G2540A), AF511526 (Porcilis PRRS vaccine),AY148221 (HMBS), AY148222 (UBE2D2), AF143818 (HPRT variant) and AF543683 (PPIA-like pseudogene). The sequence of the Austrian PRRSV-1 isolate AGS-96 (AF531277) 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 amplification 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 controls (E > 0.9, i.e. optimal) were comparable to those of the viral assays (ΔΔCt < 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 (ΔΔCt for mRNA and the corresponding minus RT control > 6 Δ).

For RNA quantification in semen specimens UBE2D2 and HMBS were analysed in more detail since in semen the differences between the Ct value for PPIA or HPRT and the corresponding minus RT sample controlling 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 positive PRRSV serostatus. Independently of the PRRSV serostatus or presence of low PRRSV RNA copy numbers (see below), the Ct 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 > 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 ORF5 copy numbers, C > 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 Arabidopsis 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 sample with 37,500 copies of the rbcL in vitro transcript, Ct 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 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 Ct 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 × 10^8 cells/ml. Duplicate Ct measurements for the 10, 20, 30 and 40 μl semen aliquots ranged between 31.3 ± 0.1 and C > 45 (no amplification). The lowest Ct values were obtained for the 30 μl and/or the 40 μl semen aliquots (Ct: 31.3 ± 0.1 to 36.7 ± 0.2, and 32.0 ± 0.5 to C > 45). No correlation was observed between Ct 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). Ct > 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.

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 $2.4\times10^6$ 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
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 mRNA) reference RNAs. Neither RNA 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 RNA 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 with PRRS viral RNA-positive or negative status (Ct ≤ 0.4).

Table 1

| Breed | PPIA mRNA | UBE2D2 mRNA | rbcL mRNA | UBE2D2 mRNA |
|-------|------------|--------------|-----------|--------------|
| P     | 32.7 ± 0.7 | 33.0 ± 0.5   | 34.0 ± 0.5 | 36.0 ± 0.7   |
| LW    | 33.0 ± 0.5 | 33.5 ± 0.6   | 34.5 ± 0.6 | 36.0 ± 0.7   |

P. Petran, L. Landrace; LW: Large White; n.a., not analysed; Ct > 45, undetectable signal.

a Negative if s/p ratio ≤ 0.4.
b Ct > 45 for PRRSV-2.
c OrFS RNA of PRRSV-1 was detected 69 days before.
d Uncate amplification.
previous study as endogenous references for human semen samples required the application of DNase I (Jussola and Bal-

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 HMBV mRNA in two samples) in expression in porcine semen RNA among the candidate refer-
gen...
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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