

ABSTRACT: Endogenous and exogenous control assays for the qualitative and quantitative detection of PRRSV RNA in the cellular fraction of semen were developed. For both control and PRRSV viral RNAs one-step real-time RT-PCR assays were optimized. Due to its low expression in seminal cells, the ubiquitin-conjugating enzyme E2D2 (UBE2D2) mRNA is an appropriate control for PRRSV molecular diagnostics, especially for the analysis of persistent infections associated with low copy numbers of viral RNA. However, in porcine seminal cells UBE2D2 mRNA was shown to be expressed at a variable but detectable extent in all samples analysed (n = 21), its use is limited to qualitative detection of PRRSV RNA. For quantitative analysis of viral RNA we used a spike with a synthetic exogenous control *rbcl* (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit from *Arabidopsis thaliana*) RNA. In principle, both reference RNAs can control for transport conditions, RNA isolation performance and amplification efficiency.

INTRODUCTION: PRRSV is one of the most economically significant pathogens in the swine industry and is endemic in most European and North American swineherds (1). The virus is an enveloped single-stranded RNA virus with a plus-sense genome (2) that belongs to the family *Arteriviridae* (3). In semen PRRSV may be shed from the bulbourethral gland (4), and can be located in immature sperm cells (5) or macrophages (6). PRRSV can be detected in semen as early as 2-3 days postinoculation (4, 7) and can be transmitted by insemination (refs. in (5)). PRRSV-infected boars show no significant clinical signs, and seroconversion and/or viremia may not be correlated with shedding of virus in semen (8).

METHOD: Ejaculates used in this study were derived from two farms. Set A (n=12 boars) were at the time of sampling tested negative with the IDEXX HerdChek PRRS ELISA. In contrast, the set B (n= 9 boars) showed a high positive serostatus upon sampling. Semen samples were subjected to RNA extraction following a silica gel based method with modifications. RNA extraction from solid tissues and serum samples was performed using commercial protocols following manufacturer's instructions. Primers and TaqMan probes in combination with Minor Groove binders (MGB probes, Applied Biosystems (9) for EU and US PRRSV viruses were based on the open reading frame 6 (ORF6), which is the most conserved protein between the two types of PRRSV virus. For RNA quantification in semen specimens two different assays were applied: (a) analysing mRNAs from four porcine endogenous controls (HPRT, HMBS, UBE2D2 and PPIA) in addition to PRRSV RNA in a multiplex real-time RT-PCR after optimization in solid porcine tissues (Fig. 1) and (b) adding the *rbcl* RNA transcript to the RNA isolation (data not shown). One-step real-time RT-PCR was performed with an ABI PRISM 7900 Sequence Detector System (Applied Biosystems).

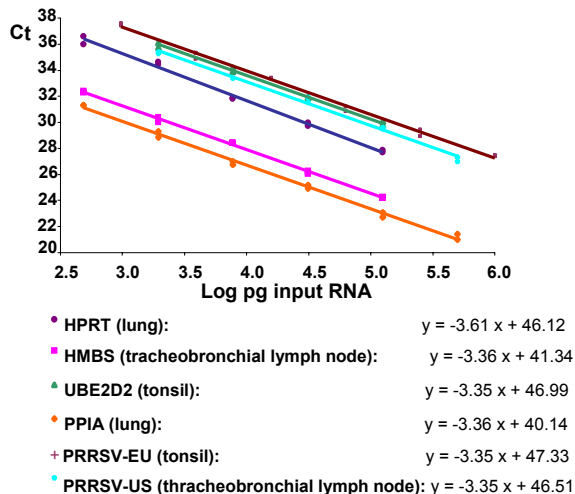


Fig. 1. One-step real-time RT-PCR of endogenous controls and PRRSV RNAs in different clinical specimens. Ct values were plotted versus the log of RNA input. The corresponding reaction efficiencies were calculated according to the equation: $E = 10^{(-1/m)} - 1$, m, slope in the regression curve $y = -m x + b$.

Boar Number (set)	Ct ₁ / Ct ₂ HMBS mRNA	Ct ₁ / Ct ₂ UBE2D2 mRNA	PRRSV serostatus	Ct for PRRSV-EU in semen ^a	Ct for PRRSV-EU in serum ^{a,d}
1 (A)	> 45/ ^b > 45	33.3/ 33.6	neg.	> 45	> 45
2 (A)	> 45/ ^b > 45	35.0/ 34.8	neg.	> 45 ^b	> 45
3 (A)	35.6/ 33.9	31.1/ 31.3	neg.	> 45 ^b	> 45
4 (A)	36.6/ > 45	36.3/ 37.1	neg.	> 45	> 45
5 (A)	36.7/ 36.7	36.3/ 35.5	neg.	> 45 ^b	> 45
6 (A)	36.6/ 35.9	35.3/ 36.2	neg.	> 45 ^b	> 45
7 (A)	> 45/ 34.8	34.3/ 34.4	neg.	> 45 ^b	35.0
8 (A)	34.2/ 33.4	31.7/ 32.7	neg.	> 45	34.9
9 (A)	30.5/ 30.3	30.6/ 32.0	neg.	> 45 ^b	> 45
10 (A)	33.5/ 33.4	33.2/ 34.0	neg.	> 45 ^b	> 45
11 (A)	> 45/ 34.5	34.8/ 35.6	neg.	> 45 ^b	34.6
12 (A)	33.3/ 32.9	34.9/ 35.5	neg.	34.6	35.6
13 (B)	n.a.	31.5/ 31.9	pos.	> 45 ^d	n.a.
14 (B)	n.a.	37.1/ 36.7	pos.	> 45 ^d	n.a.
15 (B)	n.a.	35.7/ 35.8	pos.	34.7 ^d	n.a.
16 (B)	n.a.	34.3/ 35.1	pos.	> 45 ^d	n.a.
17 (B)	n.a.	38.0/ 38.8	pos.	> 45 ^d	n.a.
18 (B)	n.a.	33.7 ^c	pos.	> 45	n.a.
19 (B)	n.a.	36.1 ^c	pos.	> 45	n.a.
20 (B)	n.a.	35.3 ^c	pos.	> 45	n.a.
21 (B)	n.a.	34.3 ^c	pos.	> 45	n.a.

Fig. 2. UBE2D2 mRNA as endogenous control for qualitative detection of PRRSV RNA. n.a., not analysed; Ct > 45, undetectable signal, ^aCt > 45 for PRRSV-2, ^bORF6 but not ORF1b RNA of PRRSV-1 was detected 69 days before, ^cunicate amplification, ^dCt > 45 for ORF1b RNA of PRRSV-1, ^enegative if s/p ratio ≤ 0.4.

DISCUSSION: Here we developed a protocol for qualitative and quantitative detection of the seminal cell-associated PRRSV RNA in relation to endogenous (UBE2D2) and exogenous (*rbcl* RNA) control RNAs. Initially the assays were optimized in porcine tissues and then applied in native semen samples derived from two sets of boars (A, B) with distinct PRRSV serostatus. Only the UBE2D2 and HMBS mRNAs were analysed in more detail. The Ct values obtained for HMBS mRNA were at or near the detection limit. In contrast, the UBE2D2 mRNA was found at a variable (up to 106-fold differences in expression) but detectable level in all samples (Fig. 2) and therefore, it is an appropriate control for qualitative detection of PRRSV in semen due to its ubiquitous expression. For quantitative detection an exogenous control (*rbcl* RNA) was spiked to the samples. We also detected viral RNA in two semen samples, one of them unexpectedly in the ELISA-negative tested group (set A). In this protocol, the use of both an endogenous and an exogenous RNA control improved the reliability of the assay since it controls for transport and storage conditions and normalizes for differences in total RNA input. Although our assay can neither differentiate between a complete viral genome and subgenomic transcripts nor gives information about the infectiousness of a sample, it is the most sensitive PRRSV assay reported so far. Therefore, RT-PCR for seminal cell-associated PRRSV is a powerful tool for managing the SPF status during quarantine programs and for routine investigations.