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Development of a one-step real-time quantitative PCR assay based on primer-probe energy transfer for the detection of porcine reproductive and respiratory syndrome virus

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A one-step real-time RT-PCR method has been developed for the simultaneous detection of both genotypes of porcine reproductive and respiratory syndrome virus (PRRSV). The assay is based on primer-probe energy transfer, and the most important advantage of this is the relative tolerance towards mutations in the target-probe region. The primers and the probe were designed using an alignment of 235 Type 1 (including all subtypes) and Type 2 PRRSV strains. According to the alignment, multiple degenerations were included in the forward and reverse primers to enable the detection of all PRRSV strains deposited in the GenBank. Specificity was tested using 37 different PRRSV strains and eight other swine pathogen viruses. The detection limit was approximately 10 copies of RNA prepared from the Lelystad virus, a European Subtype 3 virus (Belarus strain Soz-8), and an American vaccine virus (Ingelvac MLV®). One $TCID_{50}$ was the detection limit in the case of the cell cultured Lelystad virus and an American wild type isolate, respectively. The melting point analysis revealed melting point decrease, but no significant sensitivity and signal loss in the presence of numerous (up to five) target-probe mismatches, indicating the capability of tolerating even more mutations. The method was suitable for the detection and quantitation of phylogenetically divergent strains and can serve as a robust, high throughput tool for molecular diagnosis of the PRRSV.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single stranded RNA virus of the *Arteriviridae* family, member of the order *Nidovirales* (Cavanagh, 1997). The genome of PRRSV is approximately 15 kilobases (kb) in length, with nine overlapping open reading frames (ORFs). ORFs 1a and 1b comprise more than 75% of the genome, and encode for enzymes responsible for viral replication (non-structural proteins). ORF2a, and ORFs 3–5 are coding the membrane associated glycoproteins; ORF2b and ORF6 are encoding the nonglycosilated membrane proteins and the ORF7 codes for the nucleocapsid protein (Meulenberg, 2000; Wu et al., 2001).

PRRSV strains can be classified into two distinct genotypes, and marked genetic differences can be observed between the European (Type 1) and North American (Type 2) strains (Nelsen et al., 1999). The presence of Type 2 strains is reported from many countries of Europe including Hungary (Madsen et al., 1998; Indik et al., 2005; Balka et al., 2008). Sequence analysis of Lithuanian, Belarus and Russian strains revealed the definition of four distinct subtypes among the European genotype (Stadejek et al., 2002, 2006, 2008).

For the molecular diagnosis of PRRSV different reverse transcriptase polymerase chain reaction (RT-PCR) techniques have been described including nested and non-nested methods (reviewed in Fetzer et al., 2006). However, one of the most commonly used primer pairs had been reported to amplify non-PRRSV specific sequences leading to high rate of false positive results under routine laboratory conditions (Fetzer et al., 2006), even though it will only occur when the given protocol is not followed exactly (Oleksiewicz, personal communication).

Real-time RT-PCR methods using SYBR Green and TaqMan® chemistries have also been reported for the detection of both genotypes (Egli et al., 2001; Wasilk et al., 2004; Kleiboecker et al., 2005; Lurchachaiwong et al., 2008; Martínez et al., 2008), TaqMan® methods however require a perfect match between the probes and the targeted region, otherwise a significant decrease in the sensitivity of the system may occur (Bustin, 2000).

Considering that PRRSV has marked genetic variability and is one of the most rapidly evolving RNA virus, the aim of the study was to develop a sensitive, and accurate real-time RT-PCR method

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that is able to detect and quantify different PRRSV strains from both genotypes, and unlike the above mentioned molecular amplification methods, can still be effective in the presence of possible nucleotide substitutions.

The primer-probe energy transfer (PriProET) uses a Texas Red labeled oligoprobe in its 3′ end, which is designed to attach next to the 5′ end FAM (6-carboxyfluorescein) labeled reverse primer. Excitation of FAM with 470 nm wavelength light causes FRET (fluorescence resonance energy transfer) to the adjacent Texas Red acceptor resulting in a 610 nm wavelength light emission. The amount of the light emitted correlates with the template present in the reaction mixture, whereas free primers and probes in the solution generate no significant light signal.

PriProET methods have been described for the detection of several swine pathogens, including foot and mouth disease virus, vesicular stomatitis virus (Rasmussen et al., 2003, 2005), swine vesicular disease virus (Hakhverdyan et al., 2006), and hepatitis E virus (Gyarmati et al., 2007).

2. Materials and methods

2.1. Primer and probe design

Primers and probes were designed with Primer Designer 4 for Windows 95, version 4.0 SciEdCentral (Scientific and Educational Software), using a multiple alignment of 235 complete and partial PRRSV sequences downloaded from GenBank (Bethesda, USA). The targeted regions were the ORF6 and ORF7, which are relatively conserved regions of the PRRS virus genomes. Blast (www.ncbi.nih.gov) analyses of the oligonucleotides were performed to determine their specificity. Sequences of the primers and the probe are shown in Table 1.

2.2. Viruses and RNA extraction

PRRSV strains used for quantitation included two, genetically distinct Type 1 strains: the Lelystad virus (LV, Subtype 1, accession number: AY588319), a Belarus strain (Soz-8, Subtype 3, accession number: DQ324720) and a Type 2 strain: Ingelvac MLV® PRRS (Boehringer, Ingelheim, Germany, accession number: AF159149). The virus used for the determination of the $TCID_{50}$ detection limit was the Lelystad virus, and the Type 2 P129 isolate (accession number: AF494042).

A wild type Hungarian PRRSV, isolate (HU17, accession number: DQ3666355) propagated on porcine alveolar macrophages was used for inoculation of the experimental pigs. The RNA was prepared using the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was stored at −80 °C until used.

2.3. PriProET RT-PCR assay

The parameters of the reaction (primer, probe concentration, forward/reverse primer ratio, annealing temperature, melting curves) were optimized by titration of the different variables in order to achieve low cycle threshold (C_t) values and high fluorescence signal. Reactions were run in a Corbett Research Rotor-Gene Real-Time Amplification instrument (RG-6000, Corbett Research, Mortlake, NSW, Australia), with a total reaction volume of 20 μl, under the following conditions: reverse transcription at 50 °C for 1 h, followed by 95 °C for 1 min, and 50 cycles of [94 °C for 15 s, 55 °C for 30 s, 75 °C for 20 s]. Melting point (T_m) analysis was used to confirm the specific amplification of the examined virus' nucleic acid, and was performed at the end of the run with a 60 s hold at 95 °C on the first step, then from 50 °C to 90 °C with 5 s holds at each step, rising by 1 °C/cycle. Fluorescence signal was collected during the annealing step after each cycle, and during the entire melting point analysis. The wavelength of the source light was 470 nm, and the emitted light signal was of 610 nm.

Table 1
Sequences of the primers and the probe used in the study.

Name	Orientation	Sequence, 5′–3′
Primer 1	Genomic	AGCCTCGTGYTGGGYGGCARA
Probe	Genomic	TCCGATGGGGAATGGCCAGCCAGTCT-(TxR)
Primer 2	Reverse	(FAM)-TCAGCAWYTGRCACAGYTGAT
Primer 3	Genomic	**TAATACGACTCACTATAGGGA**-GCCTCGTGCTGGGCGGCAAA[a]
Primer 4	Reverse	TCAGCAWYTGRCACAGYTGAT

[a] Nucleotides shown in bold letters indicate the specific T7 promoter sequence added to the forward primer. The position of the primers and the probe can be seen in Fig. 1.

2.4. Sensitivity and specificity of the assay

The sensitivity of the system was determined by using known amounts of recombinant RNA prepared from the Lelystad virus, the Ingelvac MLV®, and the Belarus strain. RNA was prepared as follows: conventional RT-PCR was carried out with primers 3 and 4 (Table 1; Primer 3 was identical to the forward primer used for PriProET, except a specific T7 promoter sequence, that was added to its 5′ end) on the three viral RNA samples (prepared with QIAmp Viral RNA Mini Kit), using Qiagen One-Step RT-PCR Kit (Qiagen, Hilden, Germany) at 52 °C annealing temperature. The amplicons were gel purified using the QiaQuick Gel Extraction kit (Qiagen, Hilden, Germany). The purified DNA samples were transcribed to RNA using MEGAscript® T7 Kit (Ambion, Austin, TX, USA). The RNA concentrations of the undiluted samples were determined by a spectrophotometric method on a Nanodrop ND 1000 instrument (Wilmington, USA), and the copy number was calculated by the Avogadro's formula. Ten-fold dilutions were prepared from each sample from a 10^{12} to 10^{0} RNA copy number/μl in RNAse free water. The PCR efficiency was determined by using the $E = 10^{(-1/s)} - 1$ formula (E: efficiency, s: slope). The results of the test of the ten-fold RNA dilutions were used to calculate the slope.

The sensitivity was also evaluated using serial dilutions of the Lelystad virus propagated on PAM (porcine alveolar macrophages) and the P129 isolate propagated in MA-104 cell line. Ten-fold dilutions were prepared in PBS (phosphate buffer saline) beginning from cell culture supernatants containing $10^5 TCID_{50}$/ml virus. RNA was prepared from the dilutions using the QIAmp Viral RNA Mini Kit and subjected to PriProET.

Specificity was tested using our PRRSV field strain collection obtained from field cases (Balka et al., 2008). Agarose gel electrophoresis was carried out on the PCR products to confirm the expected size of the amplicons. They were then sliced from the gel, purified as described previously and sequenced using an ABI PRISM 3100 automatic sequencer with the primers used for PriProET.

Selected swine pathogens were tested with the system to exclude the cross-reactivity of the PRRSV PriProET. Included in these were porcine circovirus type 2, swine influenza virus (H3N2 and H1N1), classical swine fever virus, porcine respiratory corona virus, Aujeszky's disease virus, porcine parvovirus, porcine cytomegalovirus.

2.5. Experimental samples

Five PRRSV negative, conventional 4 weeks old piglets were infected intranasally with 2–2 ml supernatant, containing $10^4 TCID_{50}$/ml of the Hungarian wild type virus HU17. Blood was collected on day 1, 2, 3, 4, 5, 6, 7 PI. On the eighth day the pigs were euthanized and subjected to pathological and histopathological examinations. Tonsil and lung samples were obtained, and

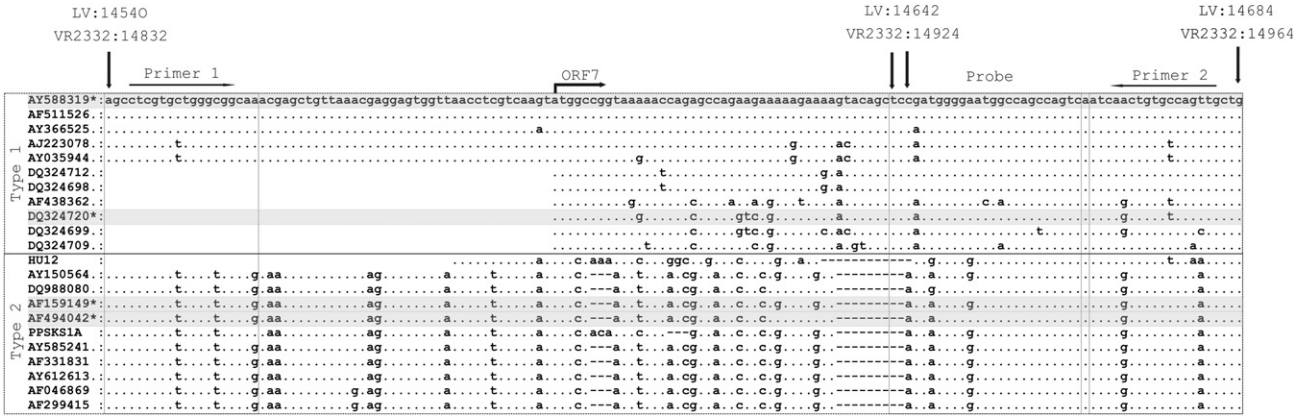

Fig. 1. Positions of the primers and the probe used for the PriProET. *Accession number of the viruses used for quantitation in the study. Grey boxes indicate their nucleotide sequence. Reference sequence is the Lelystad virus. The sequences are shown in sense orientation. (Missing information about the ORF6 sequence of 7 strains is indicated as unmarked area, whereas deletions are indicated with dashes.)

together with the sera samples they were subjected to real-time PCR. Three animals were inoculated with virus free supernatant, and involved in the study as negative controls.

3. Results

3.1. Primer and probe design

The terminal part of ORF6 for the forward primer, and the 5′ end region of ORF7 for the probe and the reverse primer were found to be suitable for FRET. The 3′ end labelled probe was designed to bind next to the 5′ end labelled reverse primer to provide adequate proximity for the FAM-Texas Red energy transfer. The forward and the reverse primers contain degenerated nucleotides to allow the attachment on both genotypes. According to Rasmussen's personal communication in order to achieve higher fluorescence signal a T overhang was added to the labelled end of the probe and the reverse primer. The probe was designed based on the Lelystad strain, and had one nucleotide mismatch on the target region of the Belarus strain, four mismatches in case of the P129 and five mismatches compared to the Ingelvac MLV® Type 2 strains. The size of the amplicon was 147nt long in case of the Type 1 strains (calculated on the Lelystad virus), and 136nt long in case of the Type 2 strains (calculated on the VR-2332 virus, accession number: AY150564). The position of the primers and the probe, can be seen on an alignment of 11 Type 1 (including Lithuanian and Belarus), and 11 Type 2 strains in Fig. 1.

3.2. PriProET RT-PCR assay

During the optimisation of the system our goal was to achieve low C_t values and the highest fluorescence signal. Based on these criteria the highest efficiency was found in case of applying asymmetric PCR conditions, by adding four times more FAM labelled reverse primer compared to the unlabelled forward (0.5 μM and 0.125 μM). The titration of the probe revealed that the right amount of probe was equal to the labelled reverse primer: 0.5 μM. The amount of dNTP was 0.4 μM in the mixture. TITANIUM™ One-Step RT-PCR Kit (Clontech Laboratories, Palo Alto, USA) containing 5′ exonuclease deficient enzyme was used for the reaction, and based on the results of several preliminary tests the Themostabilizing Reagent, the GC-Melt™, and the Oligo (dT) Primer were abolished from the reaction mixture in order to achieve higher fluorescence signal.

3.3. Sensitivity and specificity tests

The sensitivity of the system regarding the number of nucleotide copies was evaluated by using known amounts of recombinant viral RNA obtained from the Lelystad, the Soz-8, and the Ingelvac MLV® strain. The detection limit was ten copies of viral RNA in the reaction mixture and the assay allowed linear detection in the range of 10^0–10^{10} RNA copies/reaction. The standard curves were obtained with the Lelystad and the Ingelvac MLV® strain. All reactions were performed in triplicate, and the mean data of cycle threshold was

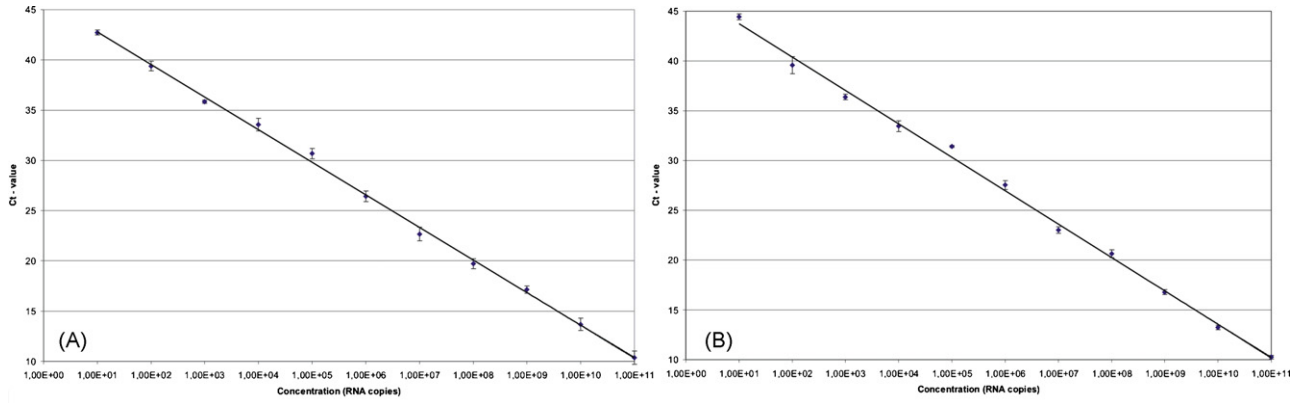

Fig. 2. Standard curves generated using 10 fold dilutions of template RNA prepared from the Lelystad (A) and Ingelvac MLV® (B) strain. (Three replicates were performed of each dilution, average values, and standard deviations are shown in the curves.) The RNA copy number is indicated on the *x*-axis, the C_t values on the *y*-axis. The R^2 (correlation effiecient) was 0.99 in both cases, whereas the *E* (efficiency) was 0.99 in case of the Lelystad virus and 1.00 in case of the the Ingelvac MLV® strain.

used to design the standard curves (Fig. 2). The PCR efficiency (E) was 0.99 in case of the Lelystad virus, and 1.00 in case of the the Ingelvac MLV® strain, whereas the correlation efficient (R^2) was 0.99 in both cases.

In case of the diluted Lelystad and P129 cell culture supernatant the detection limit was one $TCID_{50}$/ml virus which, according to the appropriate standard curves, was equal to 61 and 42 copies of viral RNA, respectively.

The assay could detect each strain that was proven to be positive by gel-based PCR. Sequence analysis of selected amplicons confirmed the amplification of PRRSV RNA. In the case of some RNA samples prepared in 2003 or before, the amplification plot was negative, but the melting point analysis could confirm the presence of the virus. Reactions performed with other (non-PRRSV) viruses gave negative results.

3.4. Melting point analysis

The melting point of the amplicons with perfect probe-target match was 76.0 °C. Amplicons with shifted melting points were sequenced using flanking primers described previously (Balka et al., 2008). As expected, mutations were confirmed to be present on the target region of the strains with lower melting points. The increasing number of mutations showed negative correlation with the melting point, but mismatches at the 5′ end had lower influence on the probe-target connection. In the case of the Ingelvac MLV® strain and the HU12 strain five mismatches were found, and the melting point of these amplicons was 66.8 °C (Figs. 1 and 3).

3.5. Experimental samples

The dynamics of the mean viral RNA quantities obtained from the sera samples of the experimental animals can be seen in Fig. 4. PRRSV RNA appeared in the sera of the animals on day 2 post-infection (PI), and after a slight, non-significant decrease the maximum quantities were observed on days 6 and 7 PI when $8–9 \times 10^5$ RNA copy/ml was present in the samples. During the pathological–histopathological examinations intralobular interstitial pneumonia in the lungs, and severe follicular hyperplasia were observed in lymphoid tissues of the challenged animals. The tonsil samples contained $1–2 \times 10^5$ RNA copy/tissue g, whereas the lung samples contained $6–9 \times 10^8$ RNA copy/tissue g. Melting point analysis performed after each PCR confirmed that the amplicons obtained from the experimental samples had the same, specific T_m (76.0 °C) as the challenge virus, confirming the successful inoculation of the animals. The negative control animals had no viral RNA in their blood or organ samples.

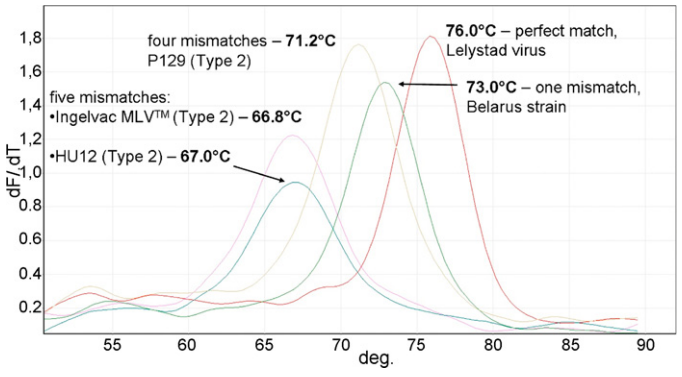

Fig. 3. Melting peaks of the different strains involved in the study with indication of the target-probe mismatches. Melting temperatures (°C) are given on the x-axis, and fluorescence (dF/dT) on the y-axis.

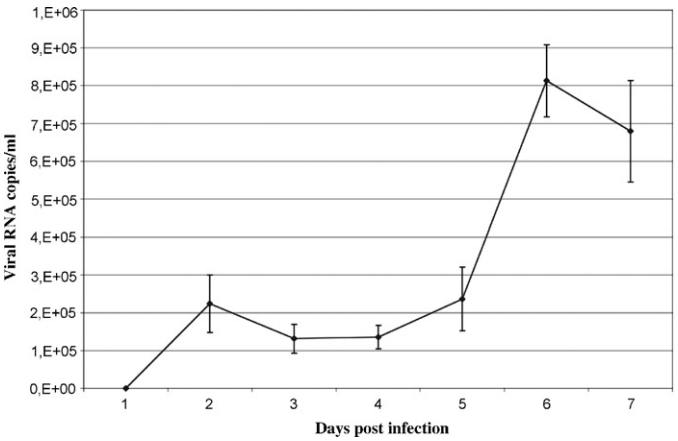

Fig. 4. Mean target RNA copy number (with standard deviation) in the serum of the experimental pigs. (Animals prior to challenge and the negative control animals had no PRRSV RNA in their blood and tissue samples.)

4. Discussion

The aim of the present study was to develop a real-time reverse transcriptase polymerase chain reaction method that is fast, sensitive, and – unlike other methods using hydrolising (TaqMan) probes – is able to tolerate mismatches in the probe region. The latter is the keypoint of this assay, since PRRSV is one of the most variable and rapidly evolving RNA viruses. In a TaqMan assay when mismatch is present in the probe binding site, the probe is displaced rather than hydrolysed, and the quencher molecule remains in the close proximity of the reporter. This can lead to signal loss, reduced sensitivity, and possible false negative results. For the PriProET reaction a 5′ exonulcease deficient enzyme is used for the polymerisation that will prevent the hydrolysis or displacement of the probe offering more stable fluorescence signal.

The TaqMan based real-time methods use single primer pair and two genotype specific probes (Egli et al., 2001; Wasilk et al., 2004; Lurchachaiwong et al., 2008) or two primer pairs and two probes (Kleiboecker et al., 2005). The PriProET assay uses only one primer-probe set to detect the full range of strains of both genotypes by tolerating numerous mutations on the target-probe area. By using fewer oligonucleotides in the reaction mixture it will be less prone to primer and primer-probe dimerisation and aspecific annealing leading to loss of fluorescence signal and sensitivity. The T overhang added to the oligonucleotides on their labelled enhanced the level of the fluorescence signal during the reactions, thus increasing the sensitivity of the system. Previous results obtained with the reverse primer and probe without the overhang showed significant lower fluorescence. The possible explanation of this phenomenon is that since T is the smallest nucleotide, it causes weaker quencher effect then the others, enhancing both the FRET and the light emission of the acceptor chromophore.

Melting point analysis performed directly after the PCR could confirm the specific hybridisation of the different PRRSV strains. By comparing the melting point of the amplicons to the perfectly matching Lelystad strain, mutations can be predicted before sequencing, however mutations at the 5′ end of the target region will have less influence on the melting point. Although the exact number of mismatches can not be stated by the degree of the temperature decrease, according to the sequences deposited in the GenBank Type 1 and Type 2 strains can be differentiated prior to sequencing the amplicons. However the probe binding site is relatively conserved, and – especially it's 5′ end – is characteristic of the PRRSV stains within a genotype (Type 2 strains have at least 4 mismatches due to a deletion and 2 conserved mutations), in the

future it can not be excluded that Type 1 strains with 4, 5 or even more mismatches may arise showing same melting points as Type 2 strains.

When the amplification plot is negative due to RNA degradation (long term storage, storage at high temperatures, autolysis etc.) and the quantitation is therefore impossible, T_m analysis can still be an effective tool to confirm the presence of the target RNA in the sample as observed by Rasmussen et al. (2003). The explanation of this finding might be the difference of the method of signal detection in case of the amplification and T_m analysis. During the amplification the absolute fluorescence signal amount is measured, whereas in case of the T_m analysis the degree of fluoresce change is calculated. In case of extremely low amounts of template RNA the absolute light signal might not reach the threshold level, whereas the signal change can be identified at the specific T_m temperature, when the probe is displaced from the template.

The PriProET method was shown to tolerate at least five mismatches in certain regions of the probe binding site (see results of the Ingelvac MLV® strain) without significant loss of sensitivity, and PCR efficiency compared to the perfect matching Lelystad strain. The melting point of the amplicon with five mismatches is high (66.8 °C), referring to a relative strong connection between the probe and the target genome. This might indicate that this PriProET method has the potential of tolerating even more mismatches in the probe binding site. The primer pairs contained multiple degenerations on the nucleotide positions that showed up to be variable on the multiple alignments. These properties enable the assay to detect broad spectrum of PRRSV strains, which, in our view, is the most important benefit of this diagnostic method.

The analysis of samples obtained from experimental infections revealed that in an acute PRRSV infection the primary replication occurs in the lungs, containing 1000 times more PRRSV RNA than the tonsil or the sera samples. PriProET can be used for following up the dynamics of the viral RNA quantities in experimental animals, and melting point analysis can confirm the specificity of the reaction by comparing the T_m of the amplicons to that of the challenge virus.

Summarising the results obtained with phylogenetically distinct PRRSV strains, and experimental samples, it is concluded that the PriProET is a robust and sensitive method that is proven to tolerate numerous target-probe mismatches making it a reliable tool to detect and quantify PRRSV.

For the future it is strongly suggested to determine and share ORF6 and ORF7 sequences of new strains from all around the world in order to provide a source of information for the constant update of the molecular diagnostic methods.

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