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Protocols

Differential detection of porcine reproductive and respiratory syndrome virus genotypes by a fluorescence melting curve analysis using peptide nucleic acid probe-mediated one-step real-time RT-PCR

Ji-Young Park[a,b,1], Seong-Hee Kim[a,1], Kyoung-Ki Lee[a], Yeon-Hee Kim[a], Bo-Yeon Moon[a], ByungJae So[a], Choi-Kyu Park[b,*]

[a] Animal Disease Diagnostic Division, Animal and Plant Quarantine Agency, Gimcheon, 39660, Republic of Korea
[b] College of Veterinary Medicine & Animal Disease Intervention Center, Kyungpook National University, Daegu, 41566, Republic of Korea

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ABSTRACT

Peptide nucleic acids (PNAs), artificially synthesized DNA analogues, hybridize strongly with DNA and are useful for fluorescence melting curve analyses (FMCA) based on the thermal denaturation of the probe-target duplex. In this study, we developed a PNA-based one-step real-time RT-PCR assay for the differential and qualitative detection of the porcine reproductive and respiratory syndrome virus genotypes PRRSV1 and PRRSV2. The specificity of the assay was analyzed in silico using previously reported primers and probes and was subsequently verified using Korean PRRSV panels and clinical samples. Seven clinical samples showing low curves with high Ct values were confirmed as negative by FMCA. The sensitivities of one-step real-time PCR for PRRSV1 and PRRSV2 were 15 and 11 copies, respectively, and the results were in 100% agreement with those of conventional RT-PCR combined with nested PCR using clinical samples. Therefore, the assay is highly specific for the detection of current PRRSV1 and PRRSV2 without non-specific amplification by FMCA.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a causative agent of reproductive failure in sows and respiratory distress in pigs of all ages, resulting in tremendous economic losses in the swine industry (Meulenberg et al., 1993; Neumann et al., 2005). The PRRSV1 (European, EU) and PRRSV2 (North American, NA) genotypes of PRRSV have diverged in two regions from the most recent common ancestor in the 1880s, sharing approximately 60% nucleotide sequence identity, with significant genetic and antigenic variation (Forsberg, 2005; Nelsen et al., 1999). In Korea, PRRSV1 emerged in the early 1990s and PRRSV2 was first isolated in 2005, after which both genotypes have co-circulated in swine herd (Kim et al., 2006; Kweon et al., 1994).

The PRRS genome is approximately 15 kb in length and is capped at the 5′-end and polyadenylated at the 3′-terminus. It contains ten open reading frames (ORFs), including ORF1a, 1b, 2a, 2b, 3, 4, 5a, 5b, 6, and 7 (Snijder and Meulenberg, 1998; Wootton et al., 2000). ORF6 and 7 encode membrane and nucleocapsid proteins, respectively, and are often used as target regions for PRRSV detection by nucleic acid-based assays (Drigo et al., 2014; Egli et al., 2001; Kleiboeker et al., 2005; Lurchachaiwong et al., 2008; Shi et al., 2016; Wu et al., 2014). In the 2000s, Korean PRRSV1 ORF7 showed a sequence identity of 88.8–99.7% with each other and 79.1–95.0% with those of other geographic regions, forming pan-European subtype I with unique clusters (I and II) (Kim et al., 2010; Lee et al., 2010). Similarly, PRRSV2 ORF7 showed a sequence identity of 86.2–100.0% with each other and 88.3–100.0% with those of other geographic regions and phylogenetic analyses indicated that Korean PRRSV2 can be classified into four groups, with Korean strains (Group 2) forming a distinct clade (Yoon et al., 2008). ORF6 sequence identities among Korean PRRSV1 in 2012 were 93.2–98.6% and between Korean strains and other non-Korean isolates were 85.6–94.4% (Lee et al., 2017). Therefore, the efficiencies of ORF6- and ORF7-based PCR should be continuously validated, since even conserved genes have high genetic diversity. The world organisation for animal health (OIE) (OIE, 2015) reported that no single reverse transcription polymerase chain reaction (RT-PCR) is capable of detecting all PRRSV genotypes, especially within the highly diverse east European subtypes of PRRSV1.

Several previous studies have reported PRRSV detection by real-

* Corresponding author.
E-mail address: parkck@knu.ac.kr (C.-K. Park).
[1] These two authors contributed equally.

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time RT-PCR (rRT-PCR), which has many advantages over conventional RT-PCR (cRT-PCR), e.g., it is less prone to contamination, allows the quantitative measurement of RNA, and is more rapid (Mackay et al., 2002). However, a low curve and high cycler threshold (Ct) value make it difficult for laboratory operators to distinguish between amplification and fluorescence artifacts. To overcome this shortcoming, we applied a probe-based fluorescence melting curve analysis (FMCA) to distinguish the characteristics of different PCR products because the melting temperature (T_m) of nucleic acids is affected by the presence of base mismatches (Elenitoba-Johnson et al., 2001; Huang et al., 2011). Peptide nucleic acids (PNA), which are artificially synthesized DNA mimics, have more favorable hybridization and biological stability than those of DNA-based probes owing to their uncharged repeating *N*-(2-aminoethyl) glycine backbone (Nielsen and Egholm, 1999; Porcheddu and Giacomelli, 2005). After the PNA probe and template DNA attach, the self-quenching dual-labeled probe elongates upon hybridization with its target and exerts fluorescence by the separation of the quencher-fluorophore pair. The fluorescent signal returns to the non-fluorescent state when the fluorophore and quencher of the PNA/DNA duplex are detached after thermal denaturation, providing information about the T_m value (Ahn et al., 2015; Huang et al., 2011). Therefore, the combination of PNA-based FMCA with rRT-PCR could minimize reaction artifacts and increase PCR specificity. The objective of the current study was to develop a one-step rRT-PCR system for the simultaneous detection of PRRSV1 and PRRSV2 by FMCA using PNA probes and degenerate primers reflecting currently conserved nucleotide regions.

2. Materials and methods

2.1. Viruses

Two reference viruses, Lelystad (GenBank No. AY035969) for PRRSV1 and LMY (GenBank No. DQ473474) for PRRSV2, were cultured with MA104 cells in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum (FBS) and antibiotic-antimycotic solution. The Lelystad and LMY viruses were titrated to $10^{5.0}$ $TCID_{50}$/mL and $10^{5.5}$ $TCID_{50}$/mL, respectively. The specificity of the assay was evaluated using the two references, classical swine fever virus LOM strain (GenBank No. EU789580), swine influenza virus VDS1 strain (GenBank No. JN043428), and porcine circovirus 2 PCK0101 strain (GenBank No. MF964235). For virus isolation, 148 tissue samples (114 lungs and 34 lymph nodes) from pigs exhibiting poor growth (40–100 days) submitted to the Animal and Plant Quarantine Agency (APQA) in 2010–2017 were used. The samples were inoculated into porcine alveolar macrophages (PAMs) harvested from the lungs of 50-day-old pigs and grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Inoculated cells were monitored daily for cytopathic effects (CPE); virus infections were confirmed by sequencing and immunofluorescence assays with N-specific monoclonal antibodies (mAb), SDOW17 (Rural Technologies, Inc., Brookings, SD, USA), and anti-NA and anti-EU (Median, Gangwon, Korea). Each genotype of PRRSV panels was established with isolated PRRSVs for the evaluation of the PCR assay developed in this study.

2.2. RT-PCR for sequencing

RNAs were extracted from the culture supernatants of PRRSV-infected PAMs using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To sequence PRRSV1 and PRRSV2, the One-step RT-PCR Kit (Qiagen) was used as described previously (Kim et al., 2010). A separate primer set for each of the two PRRSV genotypes was designed to completely sequence ORF6 and ORF7 (Supplementary Table 1). RT-PCR thermal cycling conditions for reverse transcription and amplification were as follows: 50 °C for 30 min, 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 50 s for PRRSV1 ORF6 and ORF7. All RT-PCR conditions were the same for PRRSV2 ORF6 and ORF7, except for a change in annealing temperature from 53 °C to 50 °C. Individual RT-PCR amplicons were sequenced in both directions using the gene-specific primers for subsequent analyses (Macrogen, Seoul, Korea). Individual sequences were used to obtain multiple sequence alignments and the percent nucleotide sequence identities for the PRRSV isolates were calculated using BioEdit (Ibis Biosciences, Carlsbad, CA, USA)

2.3. FMCA based on PNA probes for the detection of PRRSV by one-step rRT-PCR

2.3.1. Primer and probe design

The PRRSV sequences were aligned using CLUSTAL-X (version 1.81) and the conserved regions of the respective virus genomes were selected. Alignments of ORF6 of 100 strains of PRRSV1 and 449 strains of PRRSV2 from the NCBI database and Korean PRRSVs isolated in this study were used to identify conserved sequences within this region to design primers and probes. For color multiplexing, the probe for PRRSV1 was labeled with the 3′ quencher DABCYL and the 5′ reporter dye 6-carboxyfluorescein (FAM), and the probe for PRRSV2 was labeled with the 3′ quencher DABCYL and the 5′ reporter dye HEX. The selected primers and PNA probes were synthesized and purified by high-performance liquid chromatography in cooperation with SeaSun Biomaterials (Daejeon, Korea).

2.3.2. Conditions for one-step rRT-PCR

For one-step rRT-PCR, the Quantitative One-Step RT-PCR Kit (SeaSun Biomaterials, Daejeon, Korea) was used. One-step rRT-PCR was performed in a 20-μL reaction containing 3 μL of RNA, 0.3 μM each PRRSV forward primer, 3 μM each PRRSV reverse primer, 0.5 μM PRRSV1-FAM probe, 0.5 μM PRRSV2-HEX probe, 1 U/μL Taq polymerase, 1 U/μL RT Enzyme Mix, 2.5 mM $MgCl_2$, 200 μM dNTPs, and 7 μL of Detection Buffer. The reaction was conducted using the CFX96 Touch™Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following conditions: 50 °C for 30 min, 95 °C for 15 min, followed by 45 cycles of 95 °C for 30 s, 58 °C for 45 s, and 72 °C for 45 s. FMCA began with a denaturation step at 95 °C for 5 min, a stepwise hybridization at 75 °C, 55 °C, and 45 °C for 1 min, followed by a stepwise temperature increase to 85 °C at 1 °C/step with a 5-s interval between steps. The FMCA for optical data was performed using Bio-Rad CFX Manager 3.1.

2.4. Standard curves and validation of the assay

In vitro transcribed RNAs were generated as standards using the MEGAshortscript T7 Kit (Ambion, Foster City, CA, USA) following the manufacturer's suggestions. The transcribed RNAs were quantified (Hung-Chih et al., 2017). Ten-fold serial dilutions of the RNAs ranging from 1.5×10^7 to 1.5×10^1 PRRSV1 and 1.1×10^7 to 1.1×10^1 PRRSV2 copies were prepared and used to obtain standard curves. The intra-assay variation was determined by evaluating three samples for each genotype, e.g., 1.5×10^6, 1.5×10^4, and 1.5×10^2 PRRSV1 and 1.1×10^6, 1.1×10^4, and 1.1×10^2 PRRSV2, in three independent runs. Inter-assay variation was determined on three consecutive days with three replicates for each of the three samples.

2.5. In silico analyses based on previously reported rRT-PCR

To evaluate the efficiency of the rRT-PCR assay developed in this study, six rRT-PCR assays using TaqMan probes and primers designed to target either the ORF6 or ORF7 region were selected from published articles (Supplementary Table 2). In silico tests of primer specificity using FastPCR 6.6.34 (http://primerdigital.com) were performed with the degenerate primers and probes used in this study and the published primers and probes from selected articles using each genotype from PRRSV panels. For in silico PCR, 1 mismatch zone was allowed at the 3′

end.

2.6. Clinical samples from naturally infected piglets

A total of 100 tissue samples (lung and tonsil) were prepared from clinically poorly grown pigs (40–100 days) from 41 Korean swine farms in different geographical regions submitted to APQA in 2018. Tissues were diluted 1:10 in phosphate-buffered saline (PBS, 0.1 M, pH 7.2), grinded, vortexed, and clarified by centrifugation for 10 min at 4000 × *g* to eliminate tissue debris. Viral RNA was extracted from 300 µL of the diluted sample and used for one-step rRT-PCR for the detection and quantification of viral load. To compare detection rates, cRT-PCR (VDX® PRRSV HP MP RT-PCR; Median) combined with nested PCR (VDX® PRRSV nested PCR; Median) based on the ORF7 region, an approach that is currently used by the Veterinary Service Laboratory, was performed using RNAs from clinical samples. PCRs were conducted according to the manufacturer's instructions. Briefly, cRT-PCR was performed using 5 µL of RNA and 15 µL of RT-PCR Premix containing forward and reverse primers, for a total volume of 20 µL. cRT-PCR thermal cycling conditions were as follows: 50 °C for 30 min, 95 °C for 15 min, followed by 35 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 40 s and extension at 72 °C for 5 min. For nested PCR, 1 µL of the first cRT-PCR product was used as a template and mixed with 19 µL of nested PCR Premix containing forward and reverse primers. Cycling conditions included an initial denaturation at 94 °C for 3 min, followed by 25 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s and extension at 72 °C for 5 min. The amplified PCR product was visualized under a UV illuminator after EtBr staining.

3. Results

3.1. Virus isolation

Of the 148 samples, 96 strains were isolated and confirmed by CPE and N-specific fluorescence in PAMs (data not shown). Thirty-nine PRRSV1, 42 PRRSV2, and 15 mixed PRRSV1 and PRRSV2 were detected by the newly developed one-step rRT-PCR. The Korean PRRSV panels for each virus, including 29 strains of PRRSV1 and 36 strains of PRRSV2 excluding viruses with the same nucleotide sequences, were established for the evaluation of the PCR approach (Supplementary Table 3). Percent sequence identities of ORF6 and ORF7 in the PRRSV1 panel were 89.5–99.8% (86.4–98.3%) and 88.6–98.2% (85.7–99.2%) at the nucleotide (amino acid) level and those in the PRRSV2 panel were 86.5–99.8% (89.7–99.4%) and 83.9–99.7% (81.2–99.2%).

3.2. Evaluation of one-step rRT-PCR

The primer and probe sequences for one-step rRT-PCR were designed based on conserved regions of the terminal ORF6 of PRRSV1 and PRRSV2 (Table 1). Positive Ct and T_m values were obtained when both the prototype Lelystad and LMY were analyzed by rRT-PCR. FMCA was included to confirm the specificity of amplification and a sharp single peak was obtained within a melting temperature (T_m) range of 64 ± 2 °C. The threshold value was set to above the noise band, i.e., 50. The specificity of the test was assayed using other respiratory disease-causing viruses, such as CSFV, PCV2, and SIV, and no cross-reactivity was observed in the one-step rRT-PCR assay (data not shown).

To evaluate sensitivity, transcripts for PRRSV1 and PRRSV2 at copy numbers of 1.5×10^7 and 1.1×10^7, respectively, were used to quantify the viral RNA load. The slopes of the standard curves of PRRSV1 and PRRSV2 determined from 10-fold serial dilution stocks were −3.257 and −3.478, respectively (Fig. 1A, B). The detection limits based on these curves were 1.5×10^1 PRRSV1 and 1.1×10^1 PRRSV2 copies. One-step rRT-PCR showed a defined melting curve with a peak at 64 ± 2 °C for all dilutions, yielding positive Ct values, suggesting consistency in FMCA over a wide range of target RNA concentrations (Fig. 1C, D, E, F). The mean Ct values for the multiplex reaction were largely overlapping, differing by ≤1 cycle compared with the single reaction, indicating no interference between primers and probes.

To confirm the reproducibility and repeatability of the assay, intra-assay variation was determined with the transcript standards of 10^6, 10^4, and 10^2 copies of both viruses in three independent runs for the three samples. The intra-assay variability for PRRSV1 and PRRSV2 was low; the coefficient of variation (CV) for PRRSV1 ranged from 0.23% to 0.82% and for PRRSV2 ranged from 0.28% to 2.27% at different viral titers. Inter-assay variation was also evaluated used the transcript standards in three assay runs performed on 3 days. As indicated in Supplementary Table 4, the CV was low, with values between 0.70% and 1.64% for both viruses.

3.3. Comparison based on published rRT-PCR assays

To investigate the specificity of the primers and probes, we performed in silico PCR using the PRRSV panels established in this study. The primers and probes designed in this study provided 100% coverage across Korean PRRSVs. However, other primers and probes from previous articles resulted in 29.0–100.0% coverage for Korean RRRSVs. rRT-PCR for PRRSV1 detection reported by Egli et al. (2001) showed the lowest coverage of 29% and that for PRRSV2 by Wu et al. (2014) showed the highest coverage of 100% (Supplementary Table 5).

3.4. Application one-step rRT-PCR to clinical samples

Out of 100 samples from pig farms where wasting and respiratory syndrome was observed in 2018, 14, 26, 7, and 53 were PRRSV1, PRRSV2, mixed, and negative, respectively. The quantities of PRRSV1 and PRRSV2 RNA from clinical samples varied from about 10^2 to 10^8 copies. For mixed samples, 10^1–10^4 copies were quantified by the newly developed rRT-PCR (data not shown). The seven samples with high Ct values (Ct ≥ 38), which we analyzed for artifacts or specific positive reactions by FMCA, were negative for PRRSV (Fig. 2). The diagnostic sensitivity of one-step rRT-PCR for clinical samples was

Table 1
Primers and probes used in PNA-based one-step rRT-PCR.

Primers and probes	Sequences (5'-3')	Position		Amplicon size
		PRRSV1[a]	PRRSV2[b]	
PRRS1F	CTGCCCAYCACGTAGAAAGTGC	14,418–14,439		115bp
PRRS1R	CCTGGTACTAGAGTGCCGTT	14,513–14,532		
PRRS1 PNA	FAM-ATACGCTGTGAG-BHQ1	14,479–14,490		
PRRS2F	GGCCCCTGCCCACCACG		14,704–14,720	96bp
PRRS2R	GTAGTRGAGCCGGGACGCCG		14,780–14,799	
PRRSV2 PNA	HEX–TGATAACCACGCA-BHQ2		14,758–14,770	

[a] Nucleotide sites were based in LV4.2.1 strain (Accession No. AY588319).
[b] Nucleotide sites were based in ATCC VR-2332 strain (Accession No. U87392).

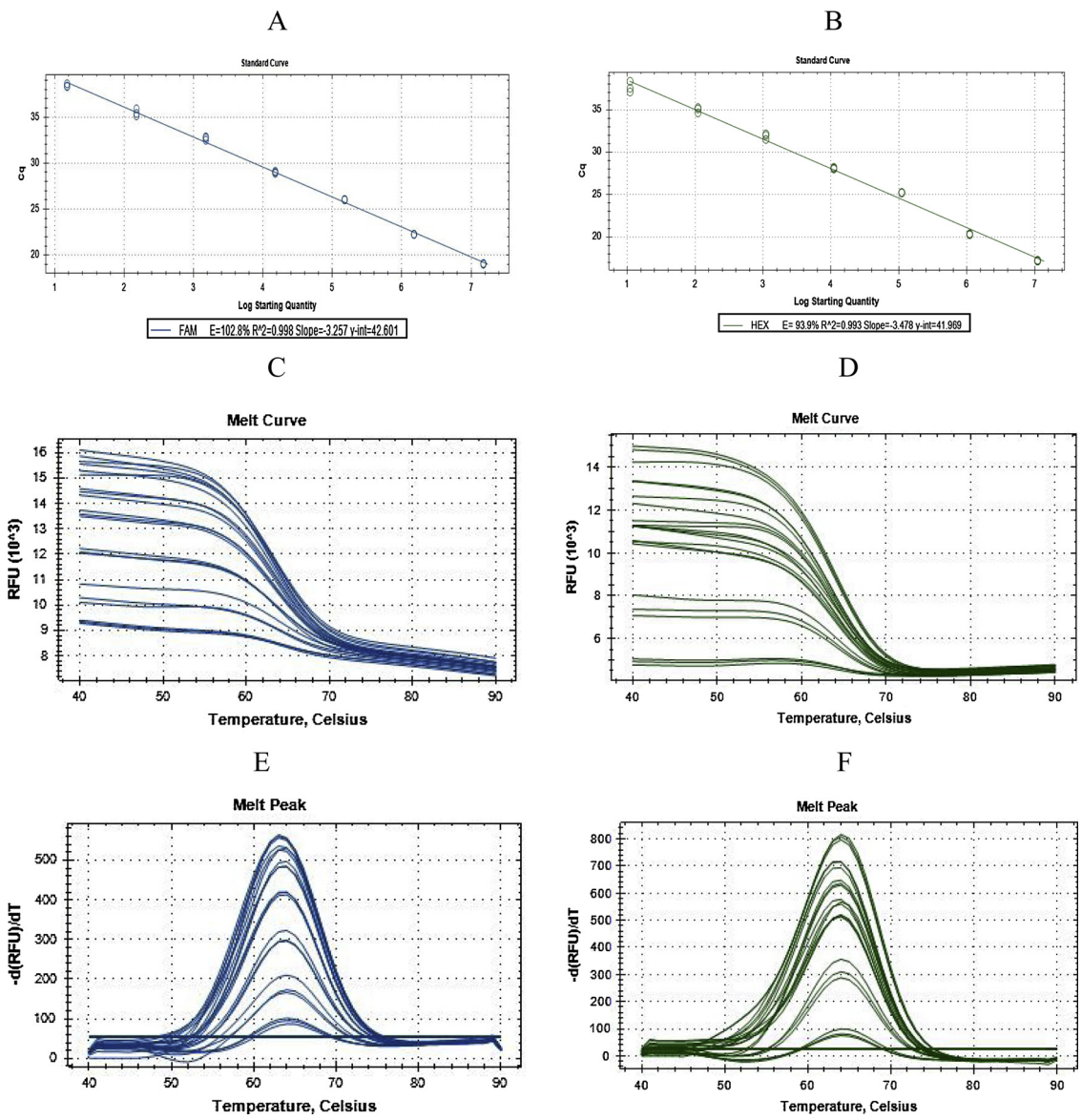

Fig. 1. Performance of FMCA using a PNA probe-based one-step rRT-PCR for PRRSV detection. Ten-fold serial dilutions ranging from 10^7 to 10^1 copies were tested in this assay. (A, B) Standard curves of each diluent were generated. (C, D) Melting curves and (E, F) melting point temperatures are shown for all template concentrations. Blue lines (A, C, E) and green lines (B, D, F) represent PRRSV1 and PRRSV2, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

100% and the specificity was 100% compared with cRT-PCR combined with nested PCR (Table 2).

4. Discussion

It is necessary to carefully determine positive and (or) negative results when performing nucleic acid-based assays for PRRSV detection owing to the genetic variation, even in the conserved regions, i.e., ORF6 and ORF7. Genetic distances of ORF6 and ORF7 between Korean PRRSV strains collected at different times were 90.2–100% and 87.3–100%, respectively, for PRRSV1 and 86.5–100% and 83.7–100% for PRRSV2. Therefore, primers and probes for PCR analyses should reflect recent current genetic variation to minimize false-negative results. Based on sequence distances, ORF6 was selected for the development of PNA-based one-step rRT-PCR owing to its greater conservation than that of the ORF7 region. In total, 630 ORF6 regions of PRRSVs available in GenBank and strains isolated in this study were aligned to design primer sets and PNA probes for the differential detection of both genotypes, since the two genotypes coexist in Korea. For the panels established in this study, ORF6 and ORF7 nucleotide sequence divergence of the PRRSV1 panel ranged from 0.2% to 11.4% and that of PRRSV2 panel ranged from 0.2% to 16.1%. Based on in silico PCR analyses using these Korean PRRSV panels, the primers and probes showed higher specificity for Korean PRRSVs and their sequences than those of previous assays, with one nucleotide mismatch with a small group of panel strains, enabling the detection of all Korean viruses used in this study. Previously published primers and probes showed low coverage for Korean PRRSVs; only 29% coverage was obtained using the primers and probe of Egli et al. (2001) for Korean PRRSV1, and mismatches between the target sequences of 10 panel viruses and the 3′ end of the primers and probe are expected to have the greatest influence on detection by in silico analyses. The sensitivity of detection was 10–15 copies for both viruses, which is similar to or over 10-fold greater than previously reported values (Drigo et al., 2014; Lurchachaiwong et al., 2008) and high inter-/intra-run repeatability was obtained, with a maximum CV of 2.3%.

PNAs have been applied for the molecular diagnosis of serval pathogens (Choi et al., 2011, 2012). PNA probes have various advantages,

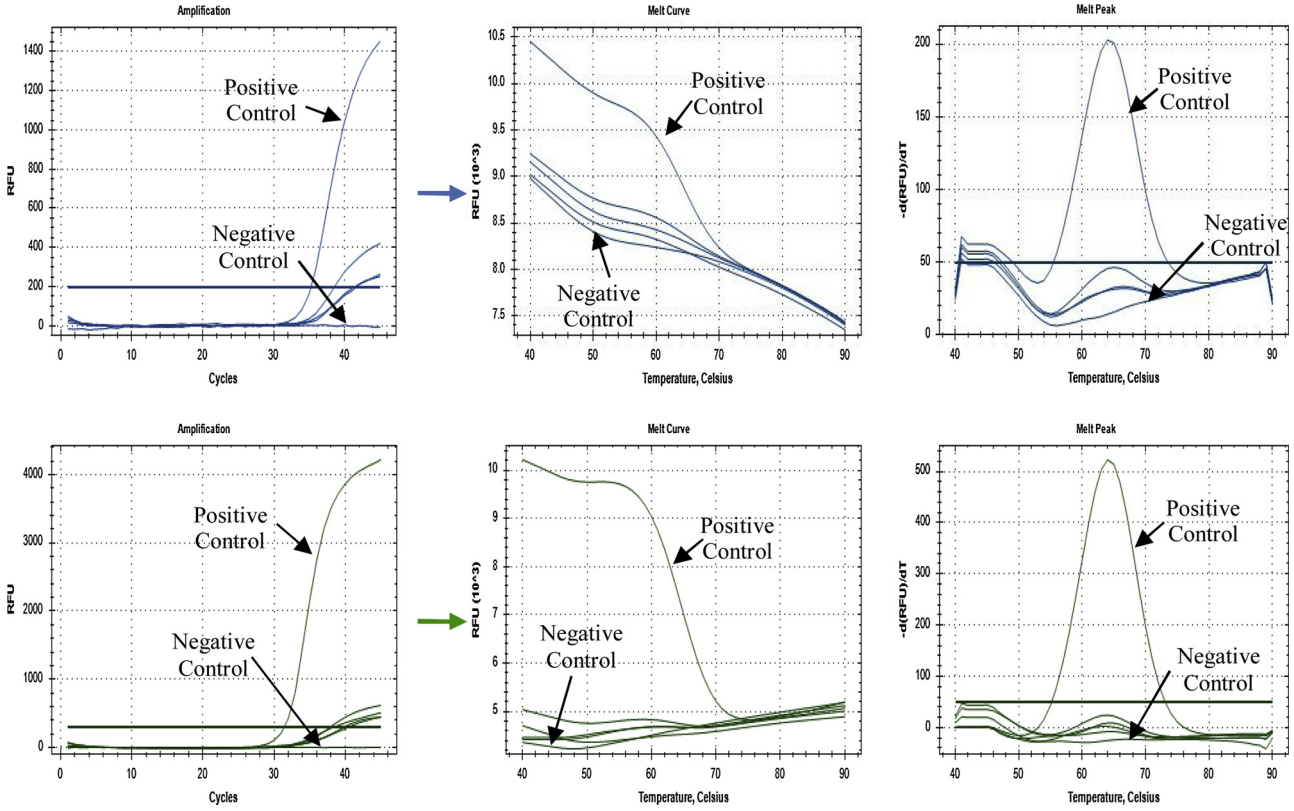

Fig. 2. Validation of seven clinical samples showing low curves with high Ct values. The upper and lower rows show the FMCA results using the PNA probe based on one-step rRT-PCR for the detection of PRRSV1 (upper row) and PRRSV2 (lower row).

Table 2
Comparison of PNA probe-based one-step rRT-PCR and conventional RT-PCR combined with nested PCR using clinical samples.

No of clinical samples	Assay		RT-PCR		Nested PCR	
			Positive	Negative	Positive	Negative
100	One-step rRT-PCR	Positive	42	5	47	0
		Negative	0	53	0	53

e.g., high affinity and sequence specificity for binding to complementary nucleic acids, since the uncharged nature of PNAs allows the formation of a strong PNA/DNA duplex. FMCA, as described in the Introduction, can be used to analyze the T_m of the PNA/DNA duplex generated from hybridization kinetics of PNAs. Therefore, in this study, considering the easy discrimination between aspecific and specific amplification by FMCA with PNA-based rRT-PCR and given the qualitative purpose of our real-time protocols, the performance of our assay is adequate for PRRSV detection.

This FMCA using PNA-based rRT-PCR for clinical samples from naturally infected pigs can detect Korean PRRSVs with high specificity. Furthermore, melting curves for the seven samples showing with high Ct values (Ct $\geq$ 38) were confirmed as negative reactions, indicating that this assay shows high diagnostic accuracy for clinical samples. The results also showed that the viral loads for PRRSV1 and PRRSV2 varied among farms. However, there was not a high degree of correlation between viral titers and mortality or the number of bacteria. The two genotypes of Northern American and European PRRSV still circulate in Korean pigs, emphasizing the need to continuously check infection statuses by monitoring with an accurate diagnostic method, which can facilitate the development of a strategy for the control of PRRSV infection. One-step rRT-PCR had a higher clinical sensitivity and the results were in 100% agreement with those of cRT-PCR combined with nested PCR. Therefore, the newly developed PCR strategy can be used in parallel with or in place of the existing cPCR, which can reduce the risk of carry-over contamination using a single closed-tube, shorten protocols, and facilitate the discrimination of specific and aspecific amplification by FMCA. In conclusion, the newly developed FMCA using PNA-based one-step rRT-PCR, in addition to the abovementioned advantages, may be an alternative to current diagnostic tools for identification, surveillance, and control in Korea.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.02.008.

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