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Rapid Detection of Type 2 Porcine Reproductive and Respiratory Syndrome Virus by a Duplex Reverse Transcription Insulated Isothermal PCR on a Field-Deployable System

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is an important porcine pathogen globally. Reverse transcription-polymerase chain reaction (RT-PCR) for PRRSV detection is an important tool for disease management and control. Clinical sensitivity of RT-PCR for PRRSV detection is compromised to a certain degree by the high genetic diversity in the PRRSV genome. A duplex RT-insulated isothermal PCR (RT-iiPCR) for the North America lineage of PRRSV (PRRSV-NA) has been developed by targeting both ORF6 and ORF7 to increase test inclusivity. In this study, its limit of detection 95% was determined to be about 5 genome equivalents per reaction by testing a serial dilution of *in-vitro* transcribed RNA. The PRRSV-NA duplex RT-iiPCR was compared with an ORF7 real-time RT-PCR (rRT-PCR) published previously for the evaluation of analytical and clinical performance. Both tests did not react with seven common swine pathogens. The two methods had similar detection endpoints for viral RNA of two PRRSV-NA isolates. Further tests with 187 swine samples showed that 14 of the 90 rRT-PCR-negative and 2 of the 97 rRT-PCR-positive samples were positive and negative by the duplex RT-iiPCR, respectively. The two methods had 91.44% agreement (95% confidential interval: 87.26 - 95.62%, $\kappa=0.83$). Repeat testing could not resolve 13 of the discrepant samples (all negative by rRT-PCR and positive by RT-iiPCR). Further RT-nested PCR analysis and DNA sequencing analysis of the ORF7 region supported that the target RNA was present in these samples. Therefore, the PRRSV-NA duplex RT-iiPCR appeared to have higher clinical sensitivity than the reference rRT-PCR. Working on a field-deployable device, the PRRSV-NA duplex RT-iiPCR has potential to serve as a fast and sensitive tool for PRRSV detection at points of need.

Keywords: PRRSV; Reverser transcription-insulated isothermal PCR; RT-iiPCR; Molecular detection; Field-deployable; Point of need

Introduction

Infection of porcine reproductive and respiratory syndrome virus (PRRSV) can lead to decreased pork production, and the costs for disease management were estimated \$664 million annually in the USA in 2011 [1,2]. PRRSV, a member of genus Arterivirus, family Arteriviridae, is an enveloped virus containing a positive single-stranded RNA of approximately 15 kb, which encodes 11 open reading frames (ORFs). ORF1a and ORF1b encode the nonstructural proteins, Nsp1 α , Nsp1 β , and Nsp2 to -12 [3]; ORF2 to ORF7 encode the structural proteins, GP2, E, GP3, GP4, GP5, M, and N [4]. PRRSV evolves rapidly and is divided into two genotypes sharing only about 60% nucleotide identity: the type 1 European lineage (PRRSV-EU, prototype the Lelystad strain) and the type 2 North American lineage (PRRSV-NA, prototype the VR-2332 strain) [5-8]. PRRSV-NA is prevalent in North America, South America, and Asia; and has also been found in Europe in recent years [8-12].

PRRSV can infect pigs of all ages. Its clinical symptoms include mild to severe respiratory syndromes in nursery-grown pigs, and reproductive failures characterized by infertility, late fetal mummification, abortions, stillbirths, and/or weak piglets [9,13]. Most importantly, PRRSV infection has been associated with the complicated porcine respiratory disease complexes [13]. Although various modified-live and inactivated vaccines are commercially available in many countries, their protection efficacy was limited to infection by PRRSV strains closely related to the vaccine strain, making the control of PRRS difficult [9,14]. Therefore, adoption of

strict biosecurity measures to help avoid or reduce the introduction and transmission of PRRSV plays an important role in the control and eradication of PRRSV; sensitive and specific detection of the etiological agent is crucial to these measures [15,16].

Several methods, including serological tests, virus isolation, and reverse transcription polymerase chain reaction (RT-PCR), are available for to help follow the status of PRRSV infection. The immunoassays, such as ELISA tests, for PRRSV-specific antibody have been commonly used to follow the immunization status of the pigs. Detection of the PRRSV by virus isolation is time-consuming and requires specific facility, technician, and cell line; furthermore, not all PRRSVs can be isolated. With high sensitivity and specificity, the RT-PCR methodology has been accepted for PRRSV detection recently [9]. The RT-PCR assays for PRRSV detection reported so far were designed to target either the ORF6 or ORF7 gene [17-22], the regions found to be the most conserved among the available PRRSV sequences [23].

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Nevertheless, PRRSV mutates quickly and has a high degree of genetic diversity [24]. False-negative results in RT-PCR tests due to sequence variations in the primer and probe target areas have been reported. Consequently, the inclusion of more than one PRRSV RT-PCR test was recommended by OIE for PRRSV detection [9,25].

A duplex RT-insulated isothermal PCR (RT-iiPCR) was developed recently for PRRSV-NA (POCKIT™ PRRSV-NA Reagent Set, GeneReach Biotech, Taichung, Taiwan) to increase the strain coverage for PRRSV detection by RT-PCR. It was designed to target two of the most conserved regions found in the ORF6 and ORF7 genes in the PRRSV genome [23]. Furthermore, in iiPCR, natural liquid convection established in a capillary tube can cycle the reaction components sequentially through different temperature zones to achieve the 3 stages (denaturation, annealing, and extension) of PCR [26-28]. Consequently, the annealing step is not done at a fixed temperature in iiPCR, allowing primers and probes to bind to sequences with minor mismatches [29]. Clinical performance of various iiPCR for various bacterial and viral pathogens in companion animals, livestock animals, and aquaculture animals, food safety, and health care has been demonstrated to be comparable to that of the reference nested PCR, real-time PCR, and/or virus isolation method [28,30-41]. In several cases, clinical sensitivity slightly higher than that of the reference real-time PCR methods was observed for targets with notable sequence variations; clinical specificity can be maintained by careful design of the primer and probe [36,42-44].

Additionally, the iiPCR works on a field-deployable device, the POCKIT™ Nucleic Acid Analyzer (POCKIT™; GeneReach, Taichung, Taiwan), which is compact (28 × 25 × 8.5 cm, W × D × H) and lightweight (2.1 kg). Automatically interpreted results are generated within one hour. The reagent is ready in a lyophilized format and minimal steps are involved in reaction assembly. Essentially, the iiPCR method is ready in a format for point-of-need applications to facilitate efficient biosecurity management and timely disease control.

In this study, analytical and clinical performance of the PRRSV-NA duplex RT-iiPCR on the POCKIT™ device was evaluated and compared to a previously published rRT-PCR which was also routinely used in a diagnostic laboratory.

Materials and Methods

Microorganisms and clinical samples

One PRRSV Taiwan isolate (CH18-2) and a VR-2332-derived PRRSV-NA vaccine (Ingelvac PRRS® MLV, Boehringer Ingelheim Vetmedica, Saint Joseph, MO, USA) were used in the sensitivity comparison study. Sequencing analysis showed that CH18-2 was closely related to the previously reported PRRSV-NA MD001 strain found in Taiwan (data not shown); it was grouped into the lineage 3 of PRRSV based on its ORF5 sequence (maximum-likelihood analysis [Tamura-Nei model] with bootstrap analysis of 1000 replicates). The exclusivity test panel included PRRSV-EU (AMERVAC® PRRS, Laboratorios Hipra S.A., Amer, Spain), porcine circovirus type 2 (PCV2; Circovac®, Merial, Lyon, France), Japanese encephalitis virus (JEV; SUIGEN® Swine Japanese Encephalitis Live Virus Vaccine-at Strain, SBC Virbac Biotech, Kaohsiung City, Taiwan), pseudorabies virus (PRV; SUIGEN® Swine Pseudorabies Gene Deleted Live Vaccine, SBC Virbac Biotech), classic swine fever virus (CSFV; Dried Lapinized Hog Cholera Vaccine, Animal Health Research Institute, Council of Agriculture, New Taipei City, Taiwan), porcine parvovirus (PPV; PORCILIS® PARVO, MSD Animal Health, Kenilworth, NJ, USA), and *Mycoplasma*

hyopneumoniae (Ingelvac MycoFLEX®, Boehringer Ingelheim Vetmedica, Saint Joseph, MO, USA). The clinical performance studies included 130 serum samples from diseased pigs and 57 postmortem lung tissues from animals with respiratory symptoms collected for surveillance or diagnostic purposes in Taiwan in 2016.

In vitro transcription

A linearized plasmid template containing the consensus sequences of ORF6 (nt 14285 - 14809, GenBank accession number KP89034) and ORF7 (nt 1 - 372, GenBank accession number JX046380) downstream of a T7-promoter was synthesized (Shanghai Genaray Biotech, Shanghai, China). The consensus sequences were derived from alignment analyses of 792 ORF6 and 1168 ORF7 sequences. From this plasmid, an artificial ORF6/ORF7 RNA was produced by *in vitro* transcription (IVT) by using the MEGAscript® T7 Kit (Thermo Fisher Scientific, Carlsbad, CA). RNA concentrations were calculated from OD260 readings measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) according to the following formula:

$$\text{No. of RNA molecules} / \mu\text{l} = \frac{\text{Avogadro number} (6.022 \times 10^{23}) \times \text{RNA concentration} (\text{g} / \mu\text{l})}{(\text{RNA molecular weight} (\text{g}))}$$

The RNA was aliquoted and stored at -80°C until use; dilutions were made in 40 ng/μl of yeast tRNA.

Nucleic extraction

Nucleic acid extraction was performed with the taco™ DNA/RNA Extraction Kit (GeneReach Biotech) on taco™ mini Nucleic Acid Automatic Extraction System (taco™ mini; Gene Reach Biotech) according to the manufacturer's instructions. For the lung tissue, 80 mg was homogenized in 500 μL phosphate-buffered saline in a taco™ Prep (GeneReach Biotech) and centrifuged at 12,000 × g for 5 min. After the wells of the extraction plate were filled with the designated buffers, 200 μL of the serum or the supernatant from homogenized lung tissues were loaded to the first well of the extraction plate. The plate was subsequently loaded into a taco™ mini device for automatic nucleic acid extraction. The nucleic acids were eluted individually in 200 μL Eluting Buffer, transferred to fresh tubes, and stored at -70°C for later use.

RRSV duplex RT-iiPCR

The PRRSV-NA duplex RT-iiPCR (POCKIT™ PRRSV Detection Kit) targeted both the ORF6 and ORF7 genes of PRRSV-NA; their amplicons were detected by the 550-nm and 520-nm channels, respectively, in the POCKIT™ device. Briefly, the lyophilized reagent was reconstituted with 50 μL Premix Buffer B and mixed with 5 μL nucleic acid extract. Subsequently, 50 μL of the final mixture were transferred to an R-tube™ (GeneReach Biotech) which was loaded into a POCKIT™ device. Qualitative results were generated by the built-in algorithm and shown on the display screen within 1 hour. Samples generating positive signals from either ORF6 or ORF7 marker were considered PRRSV positive.

PRRSV real-time RT-PCR

The reference PRRSV-NA rRT-PCR (Table 1) [17,45] was routinely used as a tool to facilitate PRRSV diagnosis at the Animal Disease Diagnostic Center, National Chiayi University. It targeted the ORF7 gene. The 25-μL reaction contained 1x PCR Reaction Buffer (BioMi, Taichung, Taiwan), 0.5 mM dNTP, 0.25 μM forward primer, 0.5 μM reverse primer, 0.2 μM probe, 0.3 μM ROX, 60 units of MMLV reverse

transcriptase (BioMi), 2 units of Taq polymerase (BioMi), and 2 μ L of sample nucleic acids. The reaction was performed on an Applied Biosystems® Step One Plus™ system (Thermo Fisher Scientific) at 42°C for 30 min followed by 40 cycles of 93°C for 15 s and 60°C for 1 min. A representative standard curve of the rRT-PCR analyses with a serial dilution of the ORF6/ORF7 IVT RNA had a linearity range between 10^2 and 10^6 copies with a slope of -3.21 (correlation coefficient, 0.99; y-intercept, 39.78; data not shown). All results that had a recorded threshold cycle (Ct) value were considered PRRSV positive.

PRRSV ORF7 RT-nested PCR and sequencing analysis

To amplifying the target region of the PRRSV-NA rRT-PCR, a degenerate RT-nested PCR (Table 1) was designed and optimized to target the highly conserved sequences found in the 212 sequences of PRRSV-NA available in the GenBank database. Briefly, the 10- μ L solution containing the sample and 1 μ M random primers was heated to 80°C for 10 min and cooled immediately on ice. Next, the 20- μ L RT reaction containing 1 \times Reaction Buffer, 0.5 mM dNTP, 200 units of MMLV reverse transcriptase (Thermo Fisher Scientific), and 10 μ L of the pretreated template was incubated at 37°C for 1 hour, 42°C for 30 min, and 94°C for 5 min. For both steps in the nested PCR, the 20- μ L reaction contained 1x PCR Reaction Buffer (BioMi), 0.125 mM dNTP, 0.25 μ M each of the forward and reverse primers, 2 units of Taq polymerase (BioMi), and 2 μ L sample. The first PCR (n-f1 and n-r1 primers, Table 1) was performed with the following program: 95°C for 30 s; 30 cycles of 95°C for 20 s, 61°C for 20 s, and 72°C for 40 s; and 72°C for 5 min. The program for the second PCR (n-f2 and n-r2 primers, Table 1) was 95°C for 30 s; 30 cycles of 95°C for 20 s, 56°C for 20 s, and 72°C for 40 s; and 72°C for 5 min. The PCR products of the expected size were verified on a 2% agarose gel stained with ethidium bromide and sent to Genomics (Taipei, Taiwan) for DNA sequencing analysis. Phylogenetic trees were constructed by using the maximum-likelihood method by the MEGA 5 software [46].

Statistical analysis

Limit of detection 95% (LOD95%) of a reaction was determined by probit analysis at 95% confidence interval by SPSS v14 (SPSS, Chicago, IL, USA). The 2 x 2 contingency tables were analyzed by kappa statistic using SPSS to determine the inter-rater agreement.

Results

Analytical sensitivity of the PRRSV-NA duplex RT-iiPCR

To assess the analytical sensitivity of the PRRSV-NA duplex RT-iiPCR, a serial dilution of the ORF6/ORF7 IVT RNA was tested. The detection rates of the 100- (10/10), 50- (20/20), 20- (20/20), and 10-copy (20/20) reactions were 100%; those of the 5-, 1-, and 0-copy ones were 95% (19/20), 25% (5/20), and 0% (0/24), respectively. Probit regression analysis determined that the LOD95% of the reaction was about 5 genome equivalents per reaction.

The analytical sensitivity of the PRRSV-NA duplex RT-iiPCR for PRRSV RNA was compared to that of the reference PRRSV-NA rRT-PCR [17,45] using serial dilutions of the nucleic acid extracts of a Taiwan isolate (CH18-2) and a VR-2332-derived vaccine. The 100% endpoints of the duplex RT-iiPCR and the rRT-PCR were at the 10^{-5} and 10^{-4} dilution, respectively, with both samples (Table 2), demonstrating that the RT-iiPCR and the rRT-PCR had similar sensitivity for the viral RNA of the PRRSV isolates.

Analytical specificity of PRRSV-NA duplex RT-iiPCR

Analytical specificity of the PRRSV-NA duplex RT-iiPCR was verified with a PRRSV-EU strain, and CSFV, PCV2, PRV, PPR, JEV, and *M. hyopneumoniae*, whose infection also causes respiratory symptoms, production failure, and/or gross lesion in the lungs on infected animals. The PRRSV-NA duplex RT-iiPCR did not detect any of these pathogens.

Clinical performance of the PRRSV-NA duplex RT-iiPCR

The PRRSV-NA duplex RT-iiPCR was compared with the reference rRT-PCR to evaluate its clinical performance for the detection of PRRSV in swine samples. Totally, 130 sera and 57 lung tissues collected in Taiwan in 2016 were tested by the two methods in parallel. Among them, 97 were positive and 90 negative by the rRT-PCR; 109 were positive and 78 negative by the duplex RT-iiPCR (Tables S1 and 3a). Two of the 97 rRT-PCR-positive samples were negative by the RT-iiPCR; notably, 14 of the 90 rRT-PCR negative samples were positive by the duplex RT-iiPCR. Based on these results, the agreement between the two assays was 91.44% (95% confidential interval: 87.26% - 95.62%; $\kappa=0.83$). Repeating tests by the two tests were performed to help resolve the discrepancy. Three of the samples (S29, S77, and S81) were positive by both methods (rRT-PCR, 1/5 [Ct=38.78], 2/5 [Ct=39.90, 38.54], and 2/5 [Ct=37.96, 37.55], respectively; duplex RT-iiPCR, 3/5, 2/5, and 2/5, respectively); the other 13 specimens still showed discordant results after repeat testing (Table S2).

RT-nested PCR and sequencing analysis of the discrepant samples

An RT-nested PCR was established to amplify the target region of the rRT-PCR in ORF7 from the 13 samples. The results of the RT-nested PCR can help resolve the discrepancies between the results of the rRT-PCR and RT-iiPCR methods, and the amplicons obtained, if any, were subjected to further sequencing analysis. Mutations at the primer/probe binding sites can lead to false-negative results in PCR assays [9,25]. RT-nested PCR products of the expected size and ORF7 sequences (nt 2-350, Figure 1) were obtained from all 13 samples, supporting that the samples were PRRSV positive. Sequencing analysis revealed that 1-3 mismatches were found in the forward primer, 1-2 mismatches in the probe, and 0-1 mismatches in the reverse primer sequences of the rRT-PCR assay. The phylogenetic tree generated from

Reaction	Name	Nucleotide sequence (5' - 3')	position*
rRT-PCR	PRRSVf2	GGGGAATGGCCAGYCAGTCAA	14927 - 14947
	PRRSVr2	GCCAGRGGAAAATGKGGCTTCTC	15061 - 15039
	US probe	FAM-CTGGGYARGATYATCGCCAGCA-BHQ1	14964 - 14986
RT-nested PCR	n-f1	GGCCCTGCCACCAC	14704 - 14719
	n-r1	GGCARACTRAACTCCACAGYGTAAC	15208 - 15183
	n-f2	ACCACGTGAAAGTGCCG	14715 - 14732
	n-r2	AACTCCACAGTGAACCTATYCTCCC	15199 - 15174

*Nucleotide position is based on the sequence of the VR-2322 strain (GenBank accession no. U87392.3)

Table 1: Sequences of primers and probe used in the PRRSV-NA rRT-PCR and RT-nested PCR.

these nucleotide sequences suggested that the 13 PRRSV samples all belonged to the PRRSV-NA lineage (data not shown).

Discussion and Conclusion

Among the tools recommended by OIE for the detection of PRRSV (virus isolation, immunoassay, RT-PCR) [9], the PCR methodology is gaining momentum in recent years. However, testing with multiple PCR methods has been recommended to help mitigate the risks of false-negative results due to the high genetic variations found in the viral genome. The PRRSV-NA duplex RT-iiPCR was designed to amplify two of the most conserved regions found in the PRRSV-NA sequences available in the GenBank database, i.e., in ORF6 and ORF7 genes, to help boost the detection inclusivity/sensitivity of the reaction for PRRSV-NA. Notably, 13 of the 90 rRT-PCR-negative samples reacted positively in the duplex RT-iiPCR test (Table S2). The positive RT-nested PCR results and sequencing analyses of these samples provided evidences for the presence of PRRSV RNA in the samples. After integrating the results of all tests, 111 PRRSV-positive samples were found; all were positive by the duplex RT-iiPCR and 13 of them were negative by the rRT-PCR (Tables 3b), implying that the duplex RT-iiPCR had higher clinical sensitivity (inclusivity) than the reference rRT-PCR. The fact that the annealing step was not carried out at a specific temperature may have also allowed the reaction to tolerate sequence mismatches to some extent in iiPCR.

Although the reference rRT-PCR contained degenerate primers and probes to be inclusive for as many strains of PRRSV as possible [17], significant numbers of mutations in the forward primer (1-3

mismatches) and/or probe binding sites (1 - 2 mismatches) were found in the 13 rRT-PCR false-negative samples. Similarly, mutations at the primer/probe binding sites caused by genetic variations led to the false-negative results in certain PRRSV real-time PCR assays [9,25]. Sequence mismatches could have decreased the binding efficiency between the oligonucleotides and the target sequences (Figure 1), leading to reduction in the melting temperatures (T_m) and negative effects on the annealing step. For 12 of the 13 discrepant samples, substantial T_m reductions (~15-30°C) between the probe of the rRT-PCR and the target sequences were suggested by bioinformatics analysis (75 mM monovalent ion and 3 mM Mg^{2+} ; OLIGO 7 software; Molecular Biology Insights, Colorado Springs, CO, USA) [49]); for the one (L2) with minor probe T_m reduction (~8°C), significant T_m reductions in both primers were predicted. One base-pair mismatch in the probe binding site had substantial influence on the sensitivity of different real-time PCR tests for influenza virus and swine hepatitis E virus [47,48].

Information of the PRRSV infection status is important for PRRSV control and elimination [15]. However, issues such as time-consuming procedures, carry-over contamination, and/or expensive equipment and special technicians have limited the application of conventional RT-PCR at points of need. The sensitive and specific iiPCR/POCKITTM system described herein is a practical tool for settings with limited resources. The device can be powered on a rechargeable or car battery; the lyophilized reagent can be shipped without a cold chain and stored for up to two years in a refrigerator. Its protocols, involving only a couple of assembly steps, can be accomplished by any

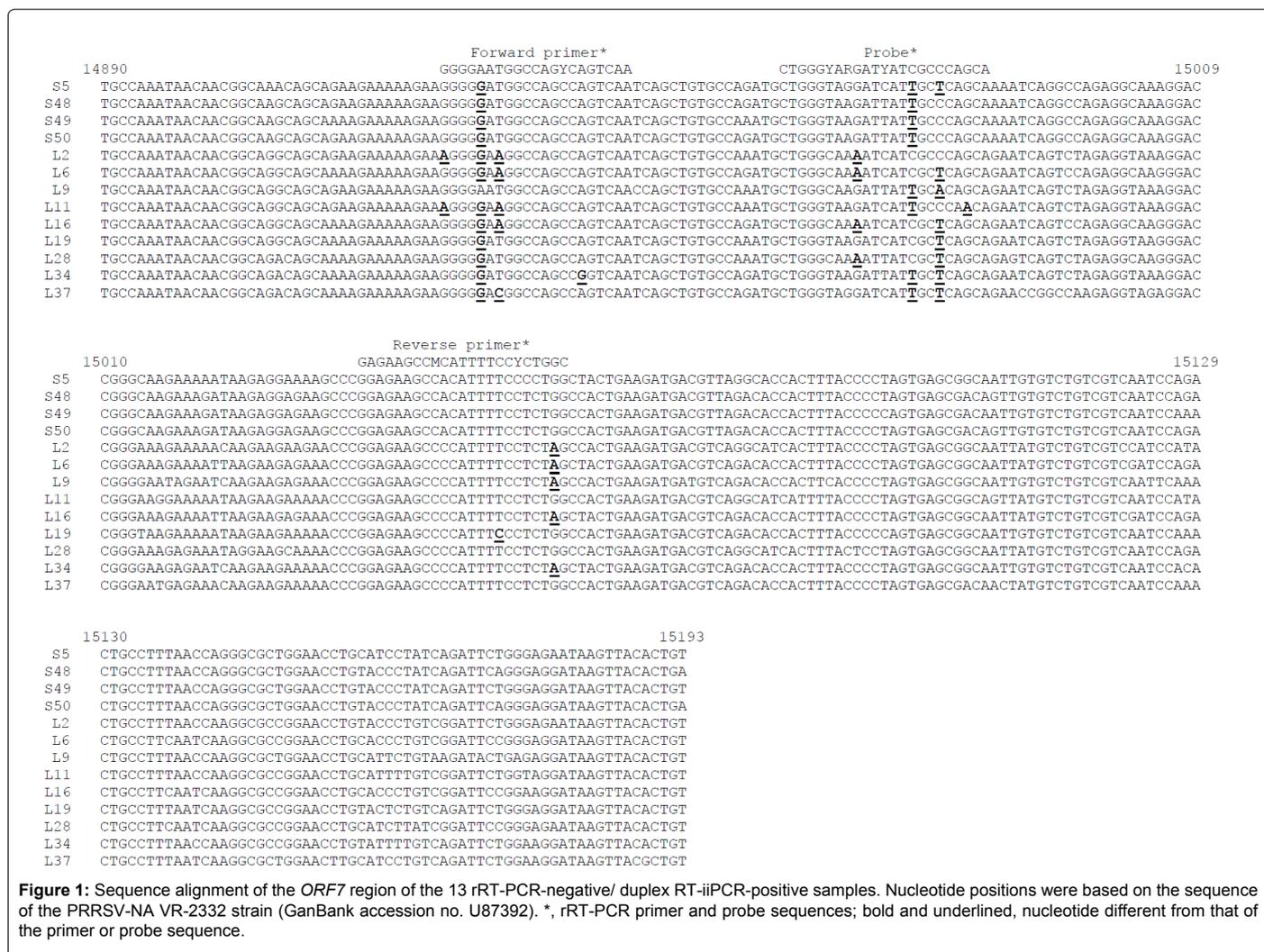
Strain	Dilution Factor	rRT-PCR (Ct)			duplex RT-iiPCR		
		test 1	test 2	test 3	test 1	test 2	test 3
VR2332-derived vaccine	10 ²	29.54	29.74	29.69	+	+	+
	10 ³	31.97	32.51	32.31	+	+	+
	10 ⁴	36	37.01	35.56	+	+	+
	10 ⁵	-	-	-	+	+	+
	10 ⁶	-	-	-	+	-	-
Taiwan CH18-2 isolate	10 ²	29.5	29.11	29.09	+	+	+
	10 ³	32.85	33.01	32.57	+	+	+
	10 ⁴	37.13	35.42	38.74	+	+	+
	10 ⁵	-	39.65	-	+	+	+
	10 ⁶	-	-	-	-	-	+

Table 2: Detection limit of the PRRSV-NA duplex RT-iiPCR: comparison with the rRT-PCR.

(a)	Variables	rRT-PCR		Total
		+	-	
	Duplex	95	14	109
	RT-iiPCR	2	76	78
	Total	97	90	187

(b)	Variables	rRT-PCR		Total
		+	-	
	Duplex	98	13	111
	RT-iiPCR	0	76	76
	Total	98	89	187

Table 3: A 2 × 2 contingency between the rRT-PCR and duplex RT-iiPCR for PRRSV-NA detection in swine samples.



users with basic training. Implemented in a closed system, the system has relatively low risks of amplicon cross-contamination. The relatively inexpensive system can be performed at locations close to or at the pen side, reducing the turn-around time to facilitate timely implementation of the measures for the control and management of PRRSV infection.

A nucleic acid extraction step before PCR is generally required to remove the reaction inhibitors from the sample matrix [49]. A field-deployable automatic nucleic acid extraction method, namely *taco*TM mini, is available to help reduce the labor costs and increase the performance consistency of the procedure. This device can also be operated on rechargeable car battery. The protocol combining the *taco*TM mini with the *POCKIT*TM device can generate qualitative test results within 2 hours with minimal hands-on time.

With great analytical sensitivity and specificity, the PRRSV-NA duplex RT-iPCR was shown to have higher clinical sensitivity than the reference rRT-PCR. Working on the field-deployable *taco*TM mini/*POCKIT*TM system in a rapid and user-friendly manner, this test can provide timely information on the status of PRRSV infection to facilitate efficient biosecurity and disease management in the swine industry.

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