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Protocols

Fluorescence resonance energy transfer combined with asymmetric PCR for broad and sensitive detection of porcine reproductive and respiratory syndrome virus 2

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ABSTRACT

With its ever-increasing viral genetic diversity, accurate diagnosis of porcine reproductive and respiratory syndrome virus (PRRSV) infection is indispensable for PRRSV control. Here, a sensitive graphene oxide (GO)-based FRET method was developed to detect PRRSV-2 based on the ability of GO to quench fluorophore by fluorescence resonance energy transfer (FRET). Using primers and a fluorophore-labeled ssDNA probe targeting a conserved region between the PRRSV M gene and 3′UTR, asymmetric PCR specifically amplified viral ssDNA that could anneal with probe to generate dsDNA only in the presence of virus. Upon exonuclease III treatment to release the probe fluorophore, which degrades dsDNA with blunt ends or recessed 3′-termini, the ssDNA annealed with other probe to generate enhanced fluorescence. This GO-based FRET assay specifically detected both classical and highly pathogenic PRRSV, with analytical sensitivity approaching 10 copies/μL, similar to that of real-time PCR but greater than that of conventional reverse transcription PCR (RT-PCR). Consistent with real-time RT-PCR detection, the assay developed here exhibited high diagnostic sensitivity for virus detection of sera from experimentally and naturally infected pigs. Thus, this novel GO-based FRET assay combined with asymmetric PCR detection is sensitive and specific and will be valuable for future PRRSV diagnosis.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-stranded enveloped RNA virus which belongs to the genus *Arterivirus*, family *Arteriviridae* and order *Nidovirales* (Lunney et al., 2016). There are two well recognized PRRSV genotypes: PRRSV-1, or European-like (prototype Lelystad), and PRRSV-2, or North American-like (prototype VR-2332) (Mardassi et al., 1994; Wensvoort et al., 1991).

Since its discovery, PRRSV has been recognized as one of the most notorious swine pathogens worldwide (Dea et al., 2000; Zimmerman et al., 1997). PRRS is characterized by respiratory syndrome and delayed growth in piglets, along with reproductive disorders in sows, leading to enormous economic losses to the global swine industry (Chand et al., 2012; Goyal, 1993; Pejsak et al., 1997). Several modified live virus (MLV) vaccines launched against both PRRSV genotypes have been licensed in various countries depending on the circulating viral genotypes in each location (Nan et al., 2017). However, the high diversity observed for recent PRRSV isolates suggests that both PRRSV-1 and PRRSV-2 are constantly evolving to adapt to existing immunity and re-emerging as new variants to cause new outbreaks continuously (Morgan et al., 2013). In China, since the first report of classical PRRSV virus in 1996 PRRSV has been constantly evolving as well, which has led to the outbreaks of highly pathogenic PRRSV (HP-PRRSV) and NADC30-like PRRSV isolates (Tian et al., 2007; Zhang et al., 2016).

Rapid and reliable diagnosis of etiological agents of infectious diseases in the early stage of infection plays a crucial role in disease control and prevention. For diagnosis of PRRSV, a variety of methods have been established for both anti-PRRSV antibody and pathogen detection. Immunohistochemical methods, enzyme-linked immunosorbent assays (ELISA), immunoperoxidase monolayer assays (IPMA), immunofluorescence assays and immunochromatographic strips have been widely used for the detection of PRRSV antigen or PRRSV-specific antibodies (Albina et al., 1992; Halbur et al., 1994; Han et al., 2012; Houben et al., 1995; Li et al., 2017; Yoon et al., 1992). Meanwhile, methods based on nucleic acid amplification, such as reverse transcriptase polymerase chain reaction (RT-PCR) (Oleksiewicz et al., 1998), highly sensitive real-time RT-PCR (Chai et al., 2013; Egli

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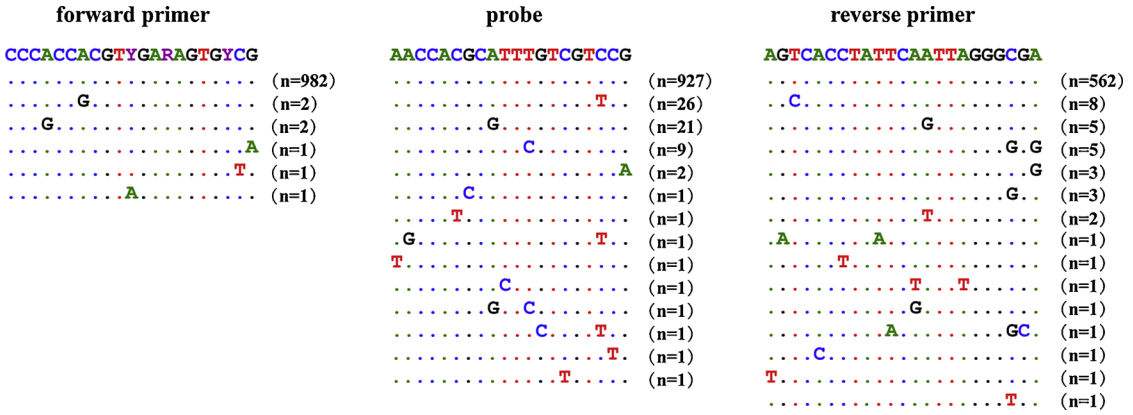

Fig. 1. Alignment of primers and probe of the GO-based FRET combined with asymmetric PCR assay with PRRSV-2 genome sequences. The sequences of forward/reverse primers and probe are shown at the top. Dots indicate nucleotides identical to the primers and probe. The numbers represent the numbers of complete genome sequences that were aligned.

et al., 2001; Kleiboeker et al., 2005; Tian et al., 2010; Wernike et al., 2012), reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Chen et al., 2010; Gao et al., 2012; Qin et al., 2009; Zhang et al., 2011) and recombinase polymerase amplification (RPA) (Wang et al., 2017; Yang et al., 2016) have been used to detect PRRSV RNA as well. However, all of the methods listed above possess various disadvantages. Therefore, an improved PRRSV diagnosis method is urgently needed.

Fluorescence resonance energy transfer (FRET) is a physical phenomenon based on the transfer of energy from a donor molecule to an adjacent acceptor molecule separated by a distance of up to 10 nm. In recent years, graphene oxide (GO), the acceptor for fluorescence quenching *via* the FRET mechanism, has been explored for biomolecular detection (Wang et al., 2011). For nucleic acid detection, GO has been used for detection of DNA molecules *via* preferential binding and quenching of fluorophore-labeled single stranded DNA (ssDNA) probes that are complementary to target DNA molecules (Huang and Liu, 2013). Subsequently, GO-based FRET diagnostic methods have been developed for detection of both viral and bacterial pathogens, such as HIV-1, *Mycobacterium tuberculosis* and white spot syndrome virus (Qaddare and Salimi, 2017; Waiwijit et al., 2015). However, the sensitivity of FRET is limited, since a single fluorophore-labeled probe is only capable of pairing with one target DNA molecule. Therefore, the application of exonuclease III (Exo III) to enhance the signal has been valuable, since Exo III preferentially catalyzes stepwise removal of mononucleotides from blunt-ended or recessed 3´-termini within dsDNA molecules, degrading the probe and releasing the fluorophore in a sequence-specific manner (Zhao et al., 2012).

In this study, a rapid, sensitive and specific assay for effective detection of PRRSV-2 RNA in pig serum samples was established based on a combination of GO-based FRET, Exo III digestion and asymmetric PCR. Concurrently, the sensitivity and specificity of this method were determined.

2. Materials and methods

2.1. Cells, virus strains and serum samples

MARC-145 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and used for PRRSV virus inoculation. PRRSV strain VR-2385 (GenBank: JX044140.1) was recovered from the infectious clone pIR-VR2385-CA by transfecting plasmids into MARC-145 cells using FuGENE® HD (Promega, Madison, WI, USA) (Ni et al., 2011). Other PRRSV virus isolates used in this study were VR-2332 (GenBank: EF536003.1), CH-1a (GenBank: AY032626), NADC30-like strain HNhx (KX766379.1) and three highly pathogenic PRRSV (HP-PRRSV) isolates: SD16 (GenBank: JX087437.1), JXA1 (GenBank: EF112445.1) and GD-HD (GenBank: KP793736.1). Viral isolates including swine influenza virus (SIV), transmissible gastroenteritis virus (TGEV), and porcine epidemic diarrhea virus (PEDV) stored in authors' laboratory were used for the specificity test.

2.2. Preparation of GO solution

Graphene oxide (GO) was purchased from Nanjing XFNANO Materials Tech Co., Ltd. (Nanjing, China). GO possesses a single-layered structure with a height of 0.8–1.2 nm made up of similar polygonal sheet structures with additional detailed wrinkled morphologies. Fourier Transform Infrared (FTIR) spectra provided by the GO supplier are in good agreement with other reports on GO (Zhu et al., 2010). To obtain a homogeneous solution, GO powder (20 mg) was dispersed in 40 mL deionized water and sonicated for 2 h following the manufacturer's instructions. After sonication, the GO solution was centrifuged (3420 x *g* for 30 min) to remove large GO layers and stored at 4 °C before use.

2.3. Design of ssDNA probe and primers for asymmetric PCR

All available M gene, 3′UTR and complete genome sequences (as of July of 2018) of PRRSV-2 isolates were downloaded from GenBank and aligned to identify conserved regions in the genome using Mafft software (Katoh and Standley, 2013). According to the alignment result, one probe was designed manually based on the conserved region of the M gene (encoded by PRRSV-ORF6) and further evaluated by software Primer Primer5. The primer set used for both asymmetric PCR and preparation of standard RNA was also designed manually and further evaluated by software Primer Primer5 (Fig. 1). Sequences of the fluorophore-labeled ssDNA probe and primers used in this study are listed in Table 1. Probe and primers were synthesized by Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

2.4. RNA extraction, reverse transcription, conventional PCR and asymmetric PCR

Total RNA was purified using TRizol LS Reagent (Invitrogen, Carlsbad, CA, USA) from 250 μL per sample of sera. RNA was resuspended in 20 μL of RNase-free water for reverse transcription by RNase H- M-MLV Reverse Transcriptase (Takara Co., Ltd., Dalian, China) according to the manufacturer's instructions.

In order to generate ssDNA for sequence-specific binding with probe, asymmetric PCR was conducted in a 50 μL reaction volume

Table 1
Sequences of probe, target cDNA and primer set used in this study.

	Sequences (5'→3')	Position[a]
FITC-Probe	FITC- AACCACGCATTTGTCGTCCG	14762-14781/M gene
Target cDNA	GGACGCCGGACGACAAATGCGTGGTTATCA	14758-14787/M gene
Forward primer	CCCACCACGTYGARAGTGYCG	14712-14732/M gene
Reverse primer	TCGCCCTAATTGAATAGGTGACT	15342-15364/3'UTR

[a] The numbers correspond to genome positions of reference sequence (NC_001961.1).

containing 25 μL reaction buffer (Takara Co., Ltd., Dalian, China), 0.4 μM reverse primer, 3 μL of cDNA template and deionized water. In the asymmetric PCR, a ratio of forward primer to reverse primer of 1:10, 1:20, 1:50, 1:100 or 1:200 was used to optimize the fluorescence detection reaction. The reaction was performed under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 40 s, and a final extension at 72 °C for 10 min. PCR products were detected by electrophoresis on 1.0% agarose gels and purified using the Cycle-Pure Kit (OMEGA, Shanghai, China).

2.5. Generation of standard RNA by in vitro transcription

The 652-bp RT-PCR product covering the region from M gene to the 3′-UTR of the PRRSV genome was obtained using the genomic RNA template of the PRRSV-JXA1 strain by RT-PCR and ligated to the pMD19-T vector. Recombinant pMD19-T vector containing RT-PCR product was linearized by *XhoI* (Takara Co., Ltd., Dalian, China) and transcribed using the AmpliCap-Max™ T7 High Yield Message Maker Kit (CELLSCRIPT, Madison, WI, USA) *in vitro* according to the manufacturer's instructions. *In vitro*-transcribed PRRSV RNA was purified using the EasyPure RNA Purification Kit (Transgen Biotech Co., Ltd., Beijing, China) and quantified using the Epoch Multi-Volume Spectrophotometer System to calculate RNA copy numbers according to the following formula: Amount (copies/μL) = [RNA concentration (ng/μL) $\times 10^{-9}$/(652 × 340)] × 6.0223×10^{23}.

2.6. Fluorescence detection and calculation

In a 100-μL reaction mixture, hybridization between probe DNA (50 nM) and target sequence was carried out in Exo III buffer for 10 min. Next, Exo III (Takara Co., Ltd., Dalian, China) was added to the mixture and incubated at 37 °C for 30 min or 4 °C for 8, 16, 24 and 32 h. Upon the addition of GO followed by incubation at 37 °C for 10 min, each reaction mixture was transferred into the 96 Well Black Assay Plate (Guangzhou Jet Bio-Filtration Co., Ltd., Guangzhou, China). Fluorescence was measured using a VICTORTM X5 Multilabel Plate Reader (PerkinElmer Inc., Waltham, MA, USA). Instrument settings were chosen as follows: CW-Lamp Energy = 16516, CW-Lamp Control: Stabilized Energy, CW-Lamp Filter: F485-Slot A5, Excitation Aperture: Normal, Emission Filter: F485-Slot A5, Emission Aperture: Normal, Counter Position: Top, Counting Time = 1 s. F0 and F represent the fluorescence intensity of the sensing system in the absence and presence of target DNA, respectively.

In order to avoid random errors, ratios of fluorescence values from PRRSV-negative serum samples (n = 200) and mean value were used to determine the cut-off value. The cut-off value for the GO-based FRET assay was set at 1 plus 3 SDs, ensuring 99% confidence for the negative serum sample that fell within this range.

The analytical sensitivity was determined by measurement of 10-fold serial dilutions (ranging from 10^1 to 10^9 copies/μL) of *in vitro*-transcribed PRRSV RNA in PRRSV-free pig serum. RNase-free water was included as negative control. After asymmetric PCR and product purification, the GO-based FRET assay was conducted. The diagnostic sensitivity of this method was evaluated by testing 32 positive serum samples collected from swine experimentally infected with PRRSV strain JXA1. Sequential serum samples collected from swine experimentally infected with PRRSV strain SD16 at 0, 4, 8, 11 and 14 dpi were also used to evaluate this method. All these samples were prepared in previous study and stored in authors' laboratory. In addition, the GO-based FRET assay was further evaluated on a total of 237 clinical serum samples.

Samples of RNA extracted from viral isolates, including SIV, TGEV and PEDV, were used for analysis of specificity. PRRSV-free serum was used as the negative control, while serum from PRRSV-infected swine served as the positive control.

2.7. Real-time RT-PCR and conventional RT-PCR

One-step real-time RT-PCR for detection of PRRSV-2 was performed using the StepOnePlus™ Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the One Step PrimeScript™ RT-PCR Kit (Takara Co., Ltd., Dalian, China) as previously described (Kleiboeker et al., 2005). In addition, a conventional RT-PCR method was performed with the same primers used in asymmetric PCR and under the same reaction volume and condition.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Differences in indicators between treatment groups and controls were assessed using the Student's *t*-test. A two-tailed *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Optimization of the GO-based FRET assay for PRRSV RNA detection

In order to obtain optimal experimental conditions to achieve maximum sensitivity and specificity, the GO-based FRET assay was optimized first. Doses of GO and Exo III and DNA hybridization and Exo III incubation times were investigated first after ssDNA probe (50 nM) and target DNA (50 nM) concentrations were set. According to the results, when the dose of Exo III was fixed at 20 U and the reaction incubated for 30 min for both DNA hybridization and Exo III incubation (Fig. 2a), the ratio of F/F0 correlated with increased concentrations of GO (ranging from 10 to 50 μg/ml). However, if the concentration of GO was further increased (50 μg/ml to 150 μg/ml), the ratio of F/F0 decreased, which may be caused by the excessive quenching effect of a high concentration of GO on fluorescence of cleaved fluorophore released from the probe, as well as high background fluorescence emitted by GO itself. Therefore, 50 μg/ml of GO was used as the optimal concentration in the assay. Using the concentration of 50 μg/ml of GO, the effect of Exo III on the assay was evaluated next. As shown in Fig. 2b, the ratio of F/F0 reach the highest value when 10 U of Exo III was used in the sensing system with incubation for 30 min per step for hybridization and enzyme digestion steps. The ratio of F/F0 decreased for Exo III dose below or above 10 U. Therefore, 10 U of Exo III was chosen as the most suitable concentration for the assay.

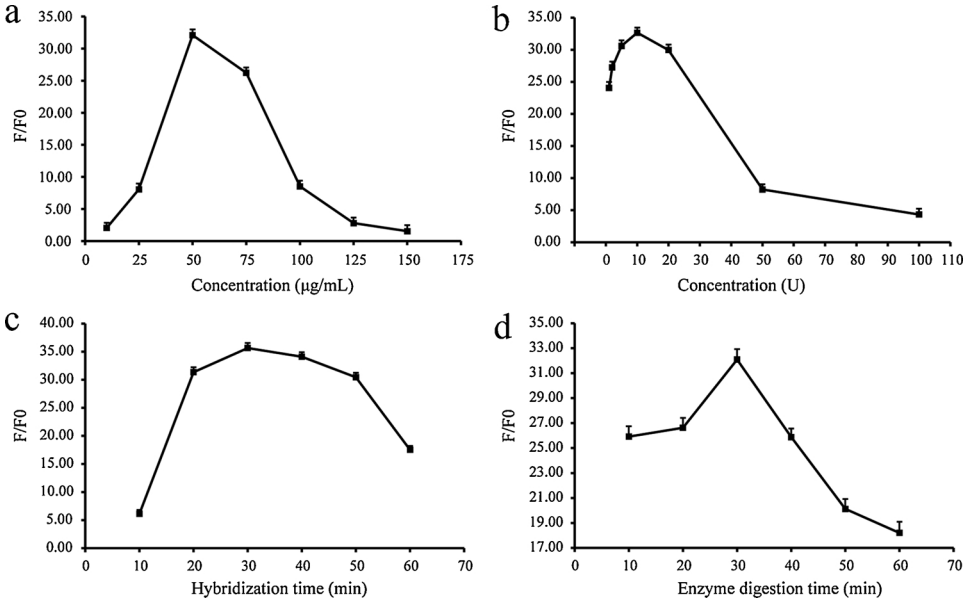

Fig. 2. Optimization of assay conditions at 50 nM ssDNA probe and 50 nM target DNA concentrations. (a) Different GO concentrations were used to quench the probe labeled fluorophore with 20 U Exo III and 30 min for both DNA hybridization and Exo III incubation; (b) Different doses of Exo III were used to degenerate the dsDNA product between ssDNA and probe with 50 μg/μL GO and 30 min for both DNA hybridization and Exo III incubation; (c) Different times for hybridization between ssDNA and probe with 50 μg/μL GO, dose of 10 U Exo III, and 30 min Exo III incubation time; and (d) Different times for degenerating the dsDNA product between ssDNA and probe with 50 μg/μL GO, 10 U Exo III, and 30 min hybridization time.

Under optimal concentrations of GO and Exo III, the hybridization time for probe to target was evaluated when the enzyme incubation time was fixed at 30 min. As demonstrated in Fig. 2c, the F/F0 ratio reached its highest value for an extended hybridization time up to 30 min. In addition, the Exo III digestion time was also optimized as well. As shown in Fig. 2d, compared with other digestion times, 30 min was the optimal digestion duration to achieve the highest value of F/F0.

3.2. Optimization of the ratio between forward primer and reverse primer for asymmetric PCR

Agarose gel electrophoresis of RT-PCR products and sequence analysis were used to confirm the size and integrity of *in vitro*-transcribed RNA (data not shown). The RNA from PRRSV-free serum diluted with transcribed PRRSV RNA was reverse transcribed into cDNA. The single stranded PCR product generated by asymmetric PCR was further purified and tested in triplicate.

RNA templates with concentrations of 10^7, 10^8 or 10^9 were used to determine the optimal ratio of forward primer to reverse primer for asymmetric PCR when the incubation condition for exonuclease treatment was at 37 °C for 30 min. As shown in Fig. 3, the variation of the ratio of forward primer to reverse primer affected the value of F/F0; the value of F/F0 reached its highest level when the forward to reverse primer ratio was 1:100. Therefore, the ratio of 1:100 of forward and reverse primers was selected for subsequent experiments.

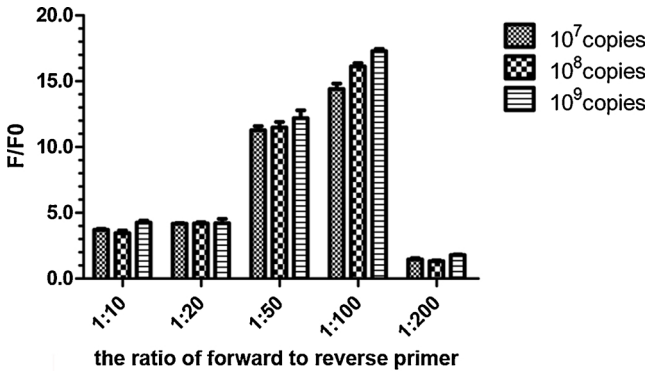

Fig. 3. Optimization of the ratio of forward primer to reverse primer for asymmetric PCR. Different ratios of forward primer to reverse primer were used with 50 nM ssDNA probe and target DNA, 50 μg/μL GO, 10 U Exo III, and 30 min incubation time at 37 ℃.

3.3. Determination of the cut-off value for GO-FRET assay combined with asymmetric PCR

Alternatively, the reaction temperature for Exo III was decreased to 4 °C and a time course study has been performed to optimize the time. As shown in Fig. 4, when the incubation condition for exonuclease treatment was at 4 °C for 24 h, the value of F/F0 was significant higher than 1 when there were 10^1 copies/μL of RNA in tested samples. However, the minimum concentration of nucleic acid is 10^2 copies/μL or 10^3 copies/μL when value of F/F0 was significant higher than 1 under the reaction condition of 37 °C for 30 min and 4 °C for 8 h, 16 h, 24 h and 32 h. Therefore, RNAs extracted from 200 pig serum samples that were negative for PRRSV were used to determine the cut-off value between positive and negative samples at 37 °C for 30 min or 4 °C for 24 h for Exo III. After RNA was reverse transcribed into cDNA, the single stranded PCR product, generated by asymmetric PCR using cDNA as template, was purified and tested using the GO-based FRET method in triplicate. The results showed that the cut-off value for the assay was 1.2 under both conditions.

3.4. Sensitivity and specificity of the GO-based FRET assay combined with asymmetric PCR for PRRSV detection

The analytical sensitivity of the GO-based FRET assay was defined as the minimum detectable number of RNA copies for which the value of F/F0 was higher than the cut-off value. When the incubation time for exonuclease treatment was 30 min at 37 °C, the value of F/F0 was 1.247 when there were 10^3 copies/μL of RNA in tested samples, a value higher than the cut-off value. Moreover, the gradient of increasing RNA concentration was consistent with F/F0 value as well (Fig. 4). When and the reaction time was extended to 24 h, the F/F0 value of the sample containing 10^1 copies/μL was 1.665, a ratio higher than the cut-off value and higher than that of the negative control; these conditions supported a nearly 2 log increase in sensitivity that was similar to the sensitivity of real-time RT-PCR (Kleiboeker et al., 2005).

The specificity of the GO-FRET assay combined with asymmetric PCR was also evaluated for samples containing nucleic acids from other swine viral isolates. As illustrated in Fig. 5, no fluorescence enhancement occurred if RNA samples were obtained from SIV, TGEV or PEDV isolates. Therefore, the GO-based FRET assay developed here was

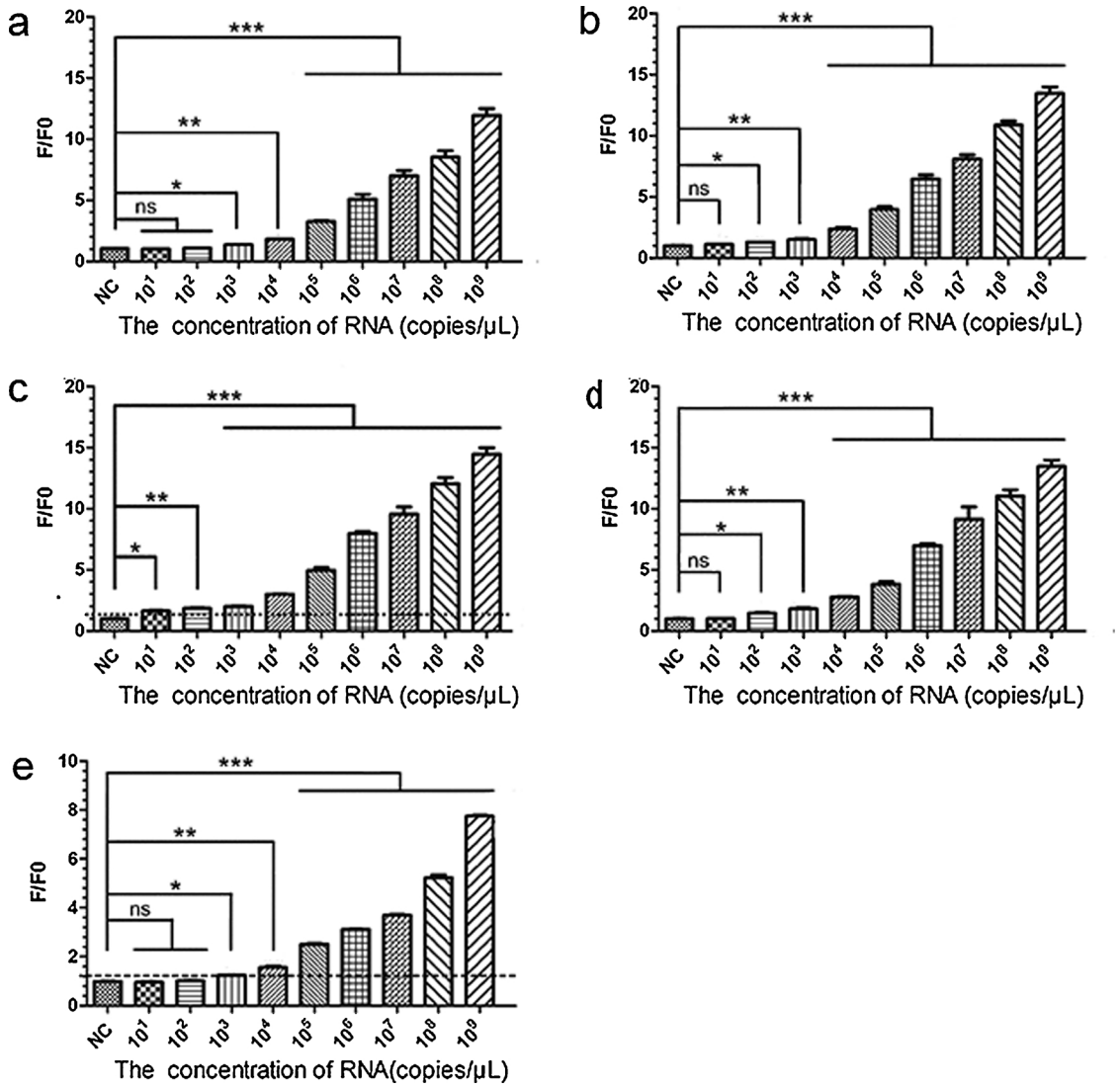

Fig. 4. The sensitivity analysis of GO-based FRET combined with asymmetric PCR using serial dilutions of *in vitro*-transcribed RNA. The reaction conditions were set at 4 °C for 8 (a), 16 (b), 24 (c), and 32 h (d) and 37 °C for 30 min (e) for GO-based FRET assay.

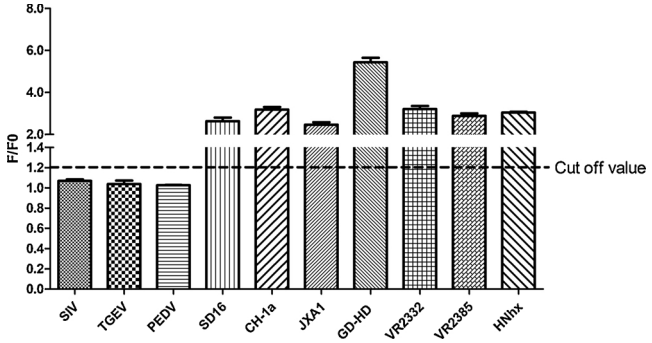

Fig. 5. The specificity of analysis of GO-based FRET combined with asymmetric PCR. Classical and highly pathogenic PRRSV strains and other swine viral pathogens were used to evaluate the specificity of GO-based FRET assay.

specific for PRRSV. Moreover, the GO-based FRET assay was effective for detection of classical PRRSV-2 isolates (CH-1a, VR2332 and VR2385), HP-PRRSV isolates (JXA1, SD16 and GD-HD) and NADC30-like strain HNhx with $TCID_{50}$ values greater than 10^5/mL (Fig. 5).

3.5. Evaluation of serum samples from experimentally infected pigs and field

Thirty-two serum samples from pigs experimentally infected with PRRSV were used to further evaluate the diagnostic sensitivity of this method. Compared with the negative control, the F/F0 values of all serum samples were above the cut-off value, suggesting the presence of viral RNA. Moreover, the GO-based FRET assay was also used to identify viral RNA from sequential serum samples under the condition of 4 °C for 24 h for Exo III. Pig sera were collected from 4 pigs at 0, 4, 8, 11 and 14 dpi after PRRSV inoculation. Viral RNA was detected in all samples except for sera collected at 0 dpi, which is consistent with real-time RT-PCR results (Table 2). However, one sample collected at 4 dpi tested negative for conventional RT-PCR. The detection results obtained with both the GO-based FRET assay and the real-time RT-PCR assay was compared. The results showed that 23 (10.1%) of 237 serum samples were positive for PRRSV RNA by the GO-based FRET, while 24 by the real-time RT-PCR assay. All the positive samples identified by the GO-based FRET assay were also positive by the real-time RT-PCR. The sample positive only detected by the GO-based FRET was retested by these two methods and the results showed the same result. The overall agreement between these two methods was 99.6%. Therefore, these data suggested that GO-based FRET combined with asymmetric PCR

Table 2
Detection of sequential serum samples collected from different dpi from 4 swines experimentally infected with PRRSV using GO-based FRET, real-time RT-PCR and conventional RT-PCR.

Swine numbers	Serum at different dpi	Methods		
		GO-based FRET	Real-time RT-PCR	conventional RT-PCR
251	0	Neg	Neg	Neg
	4	Pos	Pos	Pos
	8	Pos	Pos	Pos
	11	Pos	Pos	Pos
	14	Pos	Pos	Pos
254	0	Neg	Neg	Neg
	4	Pos	Pos	Neg
	8	Pos	Pos	Pos
	11	Pos	Pos	Pos
	14	Pos	Pos	Pos
256	0	Neg	Neg	Neg
	4	Pos	Pos	Pos
	8	Pos	Pos	Pos
	11	Pos	Pos	Pos
	14	Pos	Pos	Pos
258	0	Neg	Neg	Neg
	4	Pos	Pos	Pos
	8	Pos	Pos	Pos
	11	Pos	Pos	Pos
	14	Pos	Pos	Pos

offers a superior assay for viral RNA detection that has comparable diagnostic sensitivity to that of real-time RT-PCR.

4. Discussion

With the emergence of new pathogens or new variants of known etiological agents, accurate diagnosis of pathogens is a great concern. In China, underestimation of the genetic diversity of PRRSV strains in conjunction with the continual emergence of new PRRSV phylogenetic lineages have been frequently reported, with new variants identified as the predominant strains causing PRRS pandemics (Gao et al., 2017; Tian, 2017; Zhao et al., 2015). Therefore, greater efforts should be made to improve PRRSV surveillance.

Although PRRSV isolation based on tissue culture methods is the gold standard for PRRSV diagnosis, detection of PRRSV *via* amplification of viral genomes offers an alternative test for rapid diagnosis; methods such as RT-PCR, RT-LAMP, real-time RT-PCR and RPA, have been successfully developed for either detection or differentiation of PRRSV-1 and PRRSV-2 from classical PRRSV and HP-PRRSV strains. However, the increased heterogeneity of PRRSV isolates has resulted in mismatches between primer sequences used for nucleic acid amplification and PRRSV genome sequences obtained from field samples. Particularly, the need to employ six primers for RT-LAMP has created a big challenge for primer design due to the genetic heterogeneity of PRRSV. Therefore, these methods are not broadly applicable for detection of PRRSV and may cause false-negative results. Moreover, PCR-based detection methods have been too sensitive, causing false positive results due to contamination of PCR products generated in previous assays (Kleiboeker, 2003).

In recent years, GO-based FRET combined with PCR has served as a promising novel approach for detection of pathogenic microorganisms in both humans and animals. Recently, one report demonstrated that GO-based FRET and LAMP could be used to detect white spot syndrome virus (Waiwijit et al., 2015). Therefore, in this study, a GO-based FRET assay combined with asymmetric PCR was established for PRRSV detection. To design the primers and probe, extensive sequences of PRRSV-2 were obtained from GenBank and analyzed for conserved sequences. Based on alignment results, the forward primer and probe were designed to target the matrix gene (M), while the reverse primer was targeted to the 3′-untranslated region of the PRRSV genome, which appears to be the most conserved region among all PRRSV-2 sequences analyzed. After optimization of reaction conditions, the optional reaction concentrations of Exo III and GO were determined to be 10 U and 50 μg/ml, respectively, and the optimal hybridization time was 30 min. In this study, asymmetric PCR was conducted to generate single stranded DNA for hybridization instead of denaturing the dsDNA at high temperature. These data also suggested that this novel assay was specific for PRRSV (both classical- and HP-PRRSV strains) with no cross-reaction with other common viral porcine pathogens. Theoretically, the primers and probe designed in this study broadly consistent with most available sequences of PRRSV-2 may generate high sensitivity in the detection. In fact, whether this method can detect heterogeneous PRRSV-2 isolates should be evaluated by more isolates with large genetic differences, especially from other countries, because only seven PRRSV isolates were used in our study although these strains are dominant strains in China.

In previous reports, most assays for PRRSV RNA detection have exhibited high sensitivity. SYBR Green- or TaqMan-based real-time RT-PCR can detect PRRSV RNA copy as low as 1 copy/μL (Drigo et al., 2014). Consistent with real-time RT-PCR assays, the detection limit of the RT-LAMP assay described above was 0.001 $TCID_{50}$ or 5 copies/tube (Chen et al., 2008; Li et al., 2009). Meanwhile, the RT-RPA assay has also demonstrated a similar detection limit (Yang et al., 2016), while the sensitivity of real-time RT-PCR or RT-LAMP assays have been even as low as 10^4 copies/μL or 10^4 $TCID_{50}$/mL (Lurchachaiwong et al., 2008; Rovira et al., 2009). Here we achieved even better analytical sensitivity if an Exo III digestion step at 37 °C for 30 min was added, reaching a detection limit of 10^3 copies/μL. Notably, an even lower detection limit of 10^1 copies/μL was observed if the Exo III digestion was instead conducted at 4 °C for 24 h, attaining analytical sensitivity similar to the sensitivity of real-time RT-PCR and RT-RPA and 100-fold greater analytical sensitivity than that of conventional RT-PCR. The improved analytical sensitivity of the lower digestion temperature may be explained if at 37 °C the Exo III degraded not only the probe hybridized to the target sequence but also unbound FITC-labeled probe, which increased the background of the negative control and reduced the ratio of F/F0. In contrast, at 4 °C Exo III digestion of unbound FITC-conjugated probes was largely absent, resulting in increased F/F0 and profound improvement of the detection limit.

The application of this assay for serum samples collected from pigs experimentally infected with PRRSV and filed clinical serum samples was further evaluated and compared with one-step RT-PCR and conventional RT-PCR assays. The results demonstrated that the GO-based FRET assay, as well as one-step real time RT-PCR, was more diagnostic sensitive than conventional RT-PCR. Therefore, our GO-based FRET is a powerful method which is suitable for large-scale screening of PRRSV with comparable specificity to one-step real time RT-PCR.

Nevertheless, GO-based FRET combined with asymmetric PCR also had some disadvantages. First, GO-based FRET could not distinguish HP-PRRSV strains from classical PRRSV strains. Moreover, the total assay time takes 30 h, which is longer than real-time RT-PCR, RT-LAMP, RT-RPA and conventional RT-PCR assays. However, since asymmetric PCR uses cDNA as the template instead of PCR product, it is important to note that false positive results caused by template contamination from previous PCR steps could be reduced. In addition, the reagent cost of this method is 20% lower than that of the real-time RT-PCR, both of which were used in this study, and the equipment cost used in both two methods is approximately the same. Therefore, the GO-based FRET assay for PRRSV is still a promising method when all factors are considered.

In summary, the results demonstrated that GO-based FRET in combination with asymmetric PCR was successfully established and validated for PRRSV detection. This method offers comparable efficiency to other well-established methods for PRRSV RNA detection from serum samples. It is expected that this GO-based FRET strategy

could be valuable for future surveillance of PRRSV in clinical samples from a variety of sources.

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Declaration of Competing Interest

None.

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