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A multiplex real-time PCR panel assay for simultaneous detection and differentiation of 12 common swine viruses

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ABSTRACT

Mixed infection with different pathogens is common in swine production systems especially under intensive production conditions. Quick and accurate detection and differentiation of different pathogens are necessary for epidemiological surveillance, disease management and import and export controls. In this study, we developed and validated a panel of multiplex real-time PCR/RT-PCR assays composed of four subpanels, each detects three common swine pathogens. The panel detects 12 viruses or viral serotypes, namely, VSV-IN, VSV-NJ, SVDV, CSFV, ASFV, FMDV, PCV2, PPV, PRV, PRRSV-NA, PRRSV-EU and SIV. Correlation coefficients (R^2) and PCR amplification efficiencies of all singular and triplex real-time PCR reactions are within the acceptable range. Comparison between singular and triplex real-time PCR assays of each subpanel indicates that there is no significant interference on assay sensitivities caused by multiplexing. Specificity tests on 226 target clinical samples or 4 viral strains and 91 non-target clinical samples revealed that the real-time PCR panel is 100% specific, and there is no cross amplification observed. The limit of detection of each triplex real-time PCR is less than 10 copies per reaction for DNA, and less than 16 copies per reaction for RNA viruses. The newly developed multiplex real-time PCR panel also detected different combinations of co-infections as confirmed by other means of detections.
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1. Introduction

In the system of intensive swine production, syndromic diseases including respiratory, enteric, vesicular, and reproductive diseases are often causing different levels of morbidity and mortality, leading to significant economic losses (Butler et al., 2014; Garner et al., 2002; Jung and Saif, 2015; Kleiboeker, 2002; Ma et al., 2015). Within a given syndromic disease, clinical signs caused by different pathogens can be very similar. It is often difficult to identify the actual causal agents based on disease symptoms (Giammarioli et al., 2008; Haines et al., 2013; Xu et al., 2012). In addition, mixed infections by different pathogens make it more difficult to achieve accurate diagnosis (Wernike et al., 2013b).

The conventional methods for diagnosis of viral diseases were mainly based on viral isolation in cell culture, which is time consuming (Xu et al., 2012); and not all viruses can be isolated. Although microarray and next generation sequencing can provide more comprehensive diagnosis (Chiu, 2013; Jaing et al., 2008; Nikolaki and Tsiamis, 2013; Peterson et al., 2010; Takeichi et al., 2013), they both are still too expensive and with longer turnaround time when used in the field of veterinary diagnostics. Polymerase-chain reaction (PCR) based technologies have been widely used for detection of a number of porcine pathogens (Giammarioli et al., 2008; Haines et al., 2013; Hole et al., 2006; Huang et al., 2009; Liu et al., 2013; Ma et al., 2008; McMenamy et al., 2011; Rao et al., 2014; Wernike et al., 2013b; Xu et al., 2012). However, most methods were focused on a single or a few pathogens, thus multiple PCR tests have to be performed if several pathogens are involved. To increase PCR detection efficiency, regular multiplex PCR and multiplex RT-PCR have been developed, in which more than one target sequence were amplified by including several pairs of primers and the amplified targets were differentiated by different-sized ampli-

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cons through DNA electrophoresis (Cao et al., 2005; Giammarioli et al., 2008; Huang et al., 2009; Lee et al., 2007; Li et al., 2007; Liu et al., 2013). These procedures involve extra efforts for post-PCR processing, and the sensitivity is generally one log lower than real-time PCR (Jacob et al., 2012; Noll et al., 2015). Multiplex real-time PCR or multiplex real-time RT-PCR has been increasingly used for high throughput testing with faster turnaround. Several multiplex real-time PCR/RT-PCR targeting swine pathogens were reported (Baxi et al., 2006; Diallo et al., 2011; Haines et al., 2013; Hole et al., 2006; Horwood and Mahony, 2011; Huang et al., 2009; Nagarajan et al., 2010; Thonur et al., 2012; Wernike et al., 2013a, 2012). Most of these assays are focused on limited number of pathogens of a given syndrome, and not able to meet the requirements at the port of entry in many countries including China, which requires testing a wide range of common pathogens causing different syndromes, i.e., respiratory, reproductive or vesicular diseases, before entry. A more comprehensive and cost-effective panel assay that can rapidly detect these common swine pathogens has not been described.

In this study, a panel of multiplex real-time PCR/RT-PCR assay has been developed and validated using the most current viral genome sequence information. The panel can simultaneously detect and differentiate the following 12 common swine viruses or viral serotypes for vesicular, reproductive and respiratory diseases: Indiana serotype of Vesicular Stomatitis Virus (VSV-IN), New Jersey serotype of Vesicular Stomatitis Virus (VSV-NJ), Swine Vesicular Disease Virus (SVDV), Foot and Mouth Disease Virus (FMDV), Classical Swine Fever Virus (CSFV), African Swine Fever Virus (ASFV), Porcine Circovirus type 2 (PCV2), Porcine Parvovirus (PPV), Porcine Pseudorabies Virus (PRV), European type or type 1 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV-EU), North American type or type 2 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV-NA), and Swine Influenza Virus (SIV). These pathogens are divided into four groups or subpanels based on similar clinical signs: VSV-IN, VSV-NJ and SVDV are classified as one vesicular disease subpanel; PPV, PRV and PCV-2 are put into one subpanel for digestive and reproductive symptoms; PRRSV and SIV can cause respiratory disorders, and are categorized into another subpanel; FMDV, CSFV and ASFV can produce multi-system disorders and are put into one group. Nevertheless, this arrangement is only general classification and some viruses can cause multi-system or systemic diseases.

2. Materials and methods

2.1. Real-time PCR panel design

The panel is composed of four subpanels. Subpanel 1 targets three DNA viruses; Subpanels 2 and 4 each contains three RNA viruses; Subpanel 3 detects one DNA and two RNA viruses. The panel design is indicated in Table 1.

2.2. Primer and probe design

Molecular target for each pathogen was identified by literature search, and selected based on the number of available sequences. If multiple targets can be used, a target with more sequences and higher conservation level was used. Target gene sequences were obtained through BLAST search on GenBank website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All available sequences were collected and used for primer and probe designs to ensure high coverage over field strains. Downloaded sequences were aligned using CLC Main Workbench 7.0.3 (http://www.clcbio.com), and conserved regions were identified using BioEditor 1.6.1 (http://bioeditor.sdsc.edu/) prior to primer and probe designs. The primers and probes were designed in the most conserved region of the target gene that was identified from multiple sequence alignments to cover as many sequences as possible. The designed primers and probes have the coverage of 98% – 99.5% of sequences obtained from the GenBank (Table 1), as calculated by percentage of total sequences that matched at least one forward primer, one reverse primer and one probe sequences for a given virus or viral genotype. The individual real-time PCR primers and probes were designed using Primer 3.0 software with the ultimate objective of grouping the assays to form multiplex reactions as indicated in Table 1. All primers were designed with the melting temperature (Tm) of approximately 60 °C and the probes were specifically designed with Tm of approximately 63 °C. The primers and probes were purposely designed within a narrow annealing temperature range to facilitate the optimization process for multiple reactions in the panel. In addition, the predicted amplicon size was limited to 70–150 bp for each primer pair to potentially increase the reaction sensitivity. Primers and probes in each subpanel were checked for potential secondary structures and dimer formations prior to synthesis. All oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). The information of the primer and probe sequences, amplicon sizes, targeted genes, and numbers of available sequences used for the design for each virus are outlined in Table 1.

2.3. Preparation of standard control plasmids

The ORF 7 gene of PRRSV-NA and PRRSV-EU, M gene of SIV and ORF2 gene of PCV2 were amplified using QIAGEN one step RT-PCR kit (Qiagen, Valencia, CA, USA) or ExTaq PCR kit (TaKaRa, Mountain View, CA, USA) and cloned into pCR 2.1 vector using TOPO TA cloning kit (Invitrogen/Life Technologies, Grand Island, NY). All selected colonies were confirmed by sequencing. The target genes of the other 8 pathogens were synthesized and cloned into pUC57-kan vector by GeneWiz (South Plainfield, NJ, USA). Since the pUC57-kan vector does not have the T7/T3 promoter binding sites, each cloned fragment was re-amplified with the vector primer pair M13 F(-20) and M13 R(-27), and re-cloned into pCR 2.1 vector using TOPO TA cloning kit. The re-cloned fragments in pCR2.1 vector were used to produce RNA templates by in vitro transcription. All re-cloned plasmids were purified with Qiagen QIAamp plasmid Maxi Kit, and were quantified by a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The copy number of the extracted plasmids was calculated using the following formula (Huang et al., 2009):

$$\textit{Plasmid copies}/\mu L = \frac{(6.02 \times 10^{23}) \times (ng \ / \ \mu L \times 10^{-9})}{\textit{Plasmid length } (bp) \times \ 660}$$

The concentration of each plasmid was adjusted to 10^{10} viral copies/μL, and was used to make 10-fold serial dilutions to construct individual standard curves and to determine the limit of detection (LOD). For multiplex reactions, each standard plasmid was equally mixed and adjusted to concentration at 10^{10} copies/μL and the mixed plasmids were used to make 10-fold serial dilutions to construct triplex standard curves for analytical validation.

2.4. RNA preparation for RNA viruses

To build standard curves for RNA viruses, RNA samples of SVDV, VSV-IN, VSV-NJ, CSFV, and FMDV were produced using MEGAscript T7 Transcription kit (LifeTech., Cartsbad, CA, USA) from the respective pCR 2.1 plasmid which contains the T7 RNA polymerase promoter. The transcribed RNA samples were purified through

Table 1
Primers and probes used in the multiplex real time PCR/RT-PCR panel assay.

Subpanel	Virus (RNA/NDA)	Target gene	Product size	# of Sequences used	Primers/ probe	Sequences (5′-3′)
1	PRV (DNA)	gE	98 bp	138	FP	CGGTGCCTGCTGTACTACG
					RP	GGCGAGGTGAAGCTGCA
					Probe	Cy5- CGAGCCCTGCATCTACCACC-BHQ2
	PPV (DNA)	NS1	124 bp	97	FP	AGCGAGCCAACAACACCA
					RP	CACCAAAGCAGGCTCTTATGTC
					Probe	MAX-ACCAACCTGCACTTAACTCCAACA-BHQ1
	PCV2 (DNA)	ORF2	124 bp	2673	FP1	ACGGATATTGTAKTCCTGGTCGT
					RP1	CTTCCAACCMAAYAACAAAAGRAATCA
					RP2	ACTTCCAACCAAATAACAAAAGAAATCAG
					RP3	CCAACCAAACAACAAAAGAAACCA
					Probe	FAM-CAGTGCCGAGGCCTACRTG-BHQ1
2	SVDV (RNA)	5′UTR	111 bp	47	FP	TCCTCCGGCCCCTGAAT
					RP	ACACCCAAAGTAGTCGGTTCC
					Probe	MAX-CACCAGTGGGCAGTCTGTCG-BHQ1
	VSV-IN (RNA)	L	141 bp	38	FP	TGATGATGCATGATCCWGCTCT
					RP	ACACWCCTCCAATGGAAGGGT
					Probe	FAM-ACCGGGCTTGCACAGTTCTAC-BHQ1
	VSV-NJ (RNA)	L	141 bp	43	FP 1	GCTCTTTATGCATGACCCTGC
					FP 2	TGCTTTTTATGCATGACCCWGC
					RP	CGAGACAACGCCATACCACA
					Probe	Cy5-CTGGTTTGCACACCAGAACATTCA-BHQ2
3	ASFV (DNA)	VP72	145 bp	483	FP1	GCGATGATGATTACCTTTGCTTTG
					FP 2	CGATGATGATTACCTTCGCTTTGA
					RP 1	CGATACCACAAGATCAGCCGT
					RP 2	CTGATACCACAAGATCAGCCGT
					RP 3	GATACCACAAGATCGGCCGT
					Probe	MAX-CACGGGAGGAATACCAACCCAG-BHQ1
	CSFV (RNA)	5′UTR	110 bp	364	FP 1	AGCCCACCTCGAGATGCTA
					FP 2	AGCCCACCTCGATATGCTATG
					FP3	AGCTCACCTCGAGATGCTATG
					RP 1	CTATCAGGTCGTACTCCCATCAC
					RP 2	TATCAGGTCGTACCCCCATCA
					Probe	Cy5-ACGAGGGCAWGCCCAAGAC-BHQ2
	FMDV (RNA)	3D	99 bp	5888	FP1	ACTGGGTTTTACAAACCTGTGATG
					FP 2	CTGGGTTTTATAAACCTGTGATGGC
					RP 1	CCACGGAGATCAACTTCTCCT
					RP 2	TGCCACAGAGATCAACTTCTCC
					RP3	CCACGGAAATCAACTTCTCCTG
					Probe	FAM-TCTCCTTTGCACGCCGTGG-BHQ1
4	SIV (RNA)	M	83 bp	1528	FP 1	CCTGTCACCTCTGACTAAGGG
					FP 2	ATCCTGTCACCTCTGACTAAAGG
					FP 3	ATCTTGTCACCTCTGACTAAGGG
					FP 4	CCTGTCACCTCTGACCAAGG
					RP 1	CGTCTACGCTGCAGTCCTC
					RP 2	CGTCTACGCTGWAGTCCTCG
					RP 3	CGTCTACGtTGCAGTCCTCG
					Probe	Cy5-ACGCTCACCGTGCCSAG-BHQ2
	PRRSV-NA (RNA)	ORF7	111 bp	989	FP 1	CCAGCCWGTCAATCAGCTGT
					FP 2	CCAGCCGGTCAATCAGCT
					FP 3	CCAGCCAGTCAACCAGCT
					RP 1	GGCTTCTCCGGGTTTTTCTTY
					RP2	GGCTTCTCCGGGCTTTTCT
					RP3	GGGCTTCTCCGGGTTTTTATTC
					Probe 1	FAM-CGGTCCCTTGCCTCTGGAC-BHQ1
					Probe 2	FAM-CCGGTCCCTTRCCTCTRGACT-BHQ1
	PRRSV-EU (RNA)	ORF7	133 bp	779	FP 1	CCAGCCAGTCAATCAACTGTG
					FP 2	GCCAGTCAGTCAATCAACTGTG
					FP 3	CCAGCCAGTCAATCAGCTGT
					FP 4	GCCAGTCAGTCAATCAGCTGT
					RP 1	TCATCTTCWGCAGCCAGGG
					RP 2	TCATCTTCAGCAGCCAAGGG
					RP 3	TCATCTTCAGCAGCTAGGGGA
					RP4	TCATCTTCAGCAGCTAAGGGAAA
					Probe	MAX-TGATRAARTCCCAGCGCCAGC-BHQ1

*FP: Forward primer; RP: Reverse primer.

lithium chloride precipitation, and re-suspended with nuclease-free water. Whole genome RNA samples of PRRSV-EU and SIV were prepared from cultured virus isolates, and whole genome RNA were extracted from PRRSV-NA positive samples using Direct-zol RNA MiniPrep kit (ZYMO Research Corp., Irvine, CA, USA). The starting concentrations of the in vitro transcribed RNA samples were adjusted to 10^{10} copies/μL, and were used to make 10-fold serial dilutions to construct standard curves for sensitivity testing. The starting RNA concentrations of virus isolates (not tittered) and clinical samples were adjusted to achieve an initial real-time PCR Ct around 20, and 10-fold serial dilutions were further made to build the standard curves.

2.5. Singular and multiplex real-time PCR or RT-PCR protocols

All real-time PCR reactions were conducted with Bio-Rad CFX96™ Touch™ Real-time PCR Detection System. For DNA targets, iQ™ Multiplex Powermix kit (Bio-Rad, Hercules, CA, USA) was used. A volume of 20 μL PCR reaction contains 10 μL 2 × IQ Powermix, 0.8 μL primer mix (final concentration of 400 nM), 0.4 μL probe mix (final concentration of 200 nM), 1 μL DNA template, and 7.8 μL nuclease-free water. The reaction condition involved a 95 °C incubation for 5 min, followed by 45 cycles of denaturation at 95 °C for 15 s and a combined annealing and extension step at 60 °C for 45 s. Singular or multiplex real-time RT-PCR for RNA targets were carried out with Path-ID Multiplex One-Step RT-PCR Kit (Applied Biosystems/Life Technologies, Grand Island, NY, USA). Each reaction contains 10 μL 2 × Multiplex RT-PCR Buffer, 1 μL Multiplex Enzyme Mix, 0.8 μL primer mix (final concentration of 400 nM), 0.4 μL probe mix (final concentration of 200 nM), 1 μL RNA and 6.8 μL nuclease-free water. The reaction condition involved a reverse transcription step at 48 °C for 10 min and RT inactivation and denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 45s. If a subpanel contained both DNA and RNA viruses, the real-time RT-PCR protocol was used. The final results were analyzed using Bio-Rad CFX Manager 3.0 software.

2.6. Analytical sensitivity and standard curve of singular real-time assays

Cloned plasmids were used for all viruses; in addition, viral isolates, positive clinical samples or in vitro transcribed RNA were used for RNA viruses. Ten-fold serial dilutions of each standard plasmid were made to achieve concentrations from 10^{10} to 10^{00} copies/μL. Such serial dilutions were used to establish a standard curve for each target gene by plotting the threshold cycles with log dilution factors using three technical replications. Concentrations of RNA from viral isolates or clinical samples were adjusted to Ct = 20; in vitro transcribed RNAs were adjusted to 10^{10} copies/μL. They were used to make 10-fold serial dilutions to build the standard curves. The limits of detection were determined and calculated from the lowest concentration that all three replications were still generating positive signals.

2.7. Analytical sensitivity and standard curve of multiplex real-time assays

In each subpanel, three target plasmids were equally mixed and then 10-fold serial dilutions were made as described above. The dilutions were used to establish a standard curve for each triplex subpanel by plotting the threshold cycle and the log dilution factors. Detection limits of each target in the triplex real-time PCR assay were determined as described above. The same mixed templates were used to compare detection sensitivities between the triplex reaction and the individual singular reactions.

2.8. Analytical specificity and simultaneous detection of multiple virus targets

Because the 12 viruses used in this study are the most commonly seen viruses in swine production, potential cross-detection within the 12 pathogens were first measured to ensure the assay specificity. Positive control plasmids of all 12 targets were mixed in equal amounts and used for specificity analysis as the target pool. Non-target pools were prepared the same way as target pool except that the target plasmid was not included in the plasmid pools. Thus twelve of 11-plasmid pools and four of 9-plasmid pools were generated as non-target pools for singular and triplex reactions, respectively.

In addition, 91 clinical samples positive for non-target porcine pathogens collected from Molecular Diagnostic Lab at Kansas State University (USA) or Animal Quarantine Lab of Beijing Entry-Exit Inspection and Quarantine Bureau (China) were used for diagnostic specificity analysis. These 91 clinical samples include 40 positives to group A, 8 group C and 2 group B Porcine Rotavirus, 11 Epizootic Hemorrhagic Disease Virus (EHDV), 5 Atypical Porcine Pestivirus (APPV), 3 Porcine Parainfluenza Virus (PPIV1), 13 Porcine Epidemic Diarrhea Virus (PEDV), 1 Porcine Delta Coronavirus (PDCoV), and 8 Transmissible Gastroenteritis Virus (TGEv), respectively. Diagnostic specificity on the 12 target pathogens were also validated by 226 clinical samples and 4 viral isolates as described below.

2.9. Diagnostic performance of the PCR panel on clinical samples

A panel of 80 sera and 53 lung tissues diagnosed clinically as PRRSV and SIV infections, respectively, were collected and tested using both the triplex real-time RT-PCR of SIV, PRRSV-NA and PRRSV-EU subpanel and the three individual real-time RT-PCR assays. A panel of 24 tonsil tissues diagnosed as PPV infection, another 24 tonsil tissues diagnosed as PCV-2 infection and 16 lung tissues diagnosed as PRV infection, respectively, were collected and tested using the subpanel of PPV, PRV and PCV-2 as described above. Additionally, 15 lymph nodes diagnosed as FMDV infection, 14 lung tissues diagnosed as CSFV infection, and DNA samples from 4 different ASFV strains in USDA Foreign Animal Diseases Diagnostic Laboratory (FADDL) were used for the validation of the subpanel of FMDV, CSFV and ASFV. All results are compared with other methods currently used in Chinese National Reference Labs, Animal Quarantine Lab of Beijing Entry-Exit Inspection and Quarantine Bureau, USDA FADDL, and Molecular Diagnostic Lab at Kansas State University. Viral total nucleic acids (DNA or RNA) were simultaneously extracted from clinical samples with Qiagen (Valencia, CA) QIAamp Viral RNA Mini Kit according to the manufacture's protocol, and used for the newly developed assay validation.

3. Results

3.1. Analytical sensitivity of singular real-time PCR assays

Each standard plasmid containing viral target was serially diluted by 10-fold and tested as singular PCR in triplicate, and the results showed that the limit of detection (LOD) of individual real-time assay were 1–10 copy/μL, and its corresponding Ct values were 36–38. The standard curves of singular real-time assays showed that the correlation coefficients (R^2) and PCR amplification efficiencies (E) were all within the acceptable range. The R^2 were all greater than 0.99, and the E values were ranged between 90 and 110% (Table 2). Similar sensitivity results, including R^2, PCR amplification efficiency and LOD, were obtained from the singular real-time RT-PCR assays with in vitro transcribed RNAs of SVDV, VSV-IN, VSV-NJ, CSFV, and FMDV, cultured PRRSV-EU and SIV isolates, and clinical RNA samples of PRRSV-NA (Table 2), which indicated that the efficiency of the reverse transcription step did not have noticeable effects on sensitivities of the assays.

3.2. Analytical sensitivity of the PCR panel assays under multiplexed conditions

Results of triplex real-time assays using plasmids showed that the LOD remained 1–10 copies/μL, similar to that from singular assays. Although some targets showed a very little Ct increase than its corresponding singular real-time assay, R^2, PCR amplification efficiency and detection limit were basically remained

Table 2
Analytical sensitivity and limit of detection of the singular and triplex assays analyzed by standard curves using plasmid DNA or RNA as templates.

Virus	PCR Type	Plasmid DNA			RNA		
		R^2	E	LOD	R^2	E	LOD
PRV	Singular	0.998	95.20%	5	N/A	N/A	N/A
	Triplex	0.992	100.80%	6	N/A	N/A	N/A
PPV	Singular	0.991	105.00%	3	N/A	N/A	N/A
	Triplex	0.996	97.30%	5	N/A	N/A	N/A
PCV-2	Singular	0.998	90.40%	1	N/A	N/A	N/A
	Triplex	0.991	93.70%	2	N/A	N/A	N/A
SVDV	Singular	0.998	108.70%	2	0.997	94.2%	5
	Triplex	0.994	98.10%	2	0.981	91.3%	5
VSV-IN	Singular	0.997	106.90%	6	0.992	102.7%	5
	Triplex	0.997	96.10%	7	0.996	94.9%	14
VSV-NJ	Singular	0.997	103.70%	4	0.995	92.5%	5
	Triplex	0.999	98.60%	8	0.996	96.9%	10
ASFV	Singular	0.992	107.20%	1	N/A	N/A	N/A
	Triplex	0.999	95.90%	3	N/A	N/A	N/A
CSFV	Singular	0.998	90.90%	3	0.999	110.2%	6
	Triplex	0.994	91.00%	4	0.994	92.2%	16
FMDV	Singular	0.998	100.30%	5	0.998	102.9%	5
	Triplex	0.998	98.40%	5	0.982	93.6%	5
SIV	Singular	0.999	98.4%	1	0.995	94.7%	N/A
	Triplex	0.998	105.3%	3	0.996	110.3%	N/A
PRRSV-NA	Singular	0.999	99.00%	3	0.997	99.2%	N/A
	Triplex	0.997	92.50%	5	0.995	107.6%	N/A
PRRSV-EU	Singular	1.000	99.90%	2	0.990	103.9%	N/A
	Triplex	0.998	105.30%	3	0.998	103.6%	N/A

R^2: Correlation coefficient; E: PCR amplification efficiency (calculated by $E = 10^{-1/slope}-1$); LOD: Limit of detection (Copies/μL).

Table 3
Correlation of sensitivities between triplex assays and singular assays using Ct values generated with 10-fold serial dilutions of plasmid DNA or RNA.

Virus	Correlation coefficient (R^2)	
	Plasmid DNA	RNA
PRV	0.998	N/A
PPV	0.998	N/A
PCV-2	0.972	N/A
SVDV	0.995	0.982
VSV-IN	0.996	0.997
VSV-NJ	0.999	0.991
ASFV	0.985	N/A
CSFV	0.978	0.976
FMDV	0.998	0.991
SIV	0.994	0.993
PRRSV-NA	0.999	0.998
PRRSV-EU	0.998	0.975

R^2: Correlation coefficient of Ct values detected with triplex assay and singular assays using plasmid DNA or RNA samples.

the same (Table 2). Similar but slightly lower sensitivities were observed from the triplex real-time RT-PCR assays with RNA samples (Table 2). Correlation coefficient for SVDV was 0.981 and for FMDV was 0.982 when RNA were used in triplex reactions. Detection limit for some viruses were slightly higher as well (14 for VSV-IN; 10 for VSV-NJ; 16 for CSFV; Table 2). For all other targets, R^2 were greater than 0.99, and LOD were equal to or less than 10 copies per reaction.

3.3. Comparison of sensitivity levels between triplex and singular real-time assays

Mean Ct values of three replicates from both triplex and singular reactions were extracted from results described above (Sections 3.1 and 3.2), and used for a linear regression analysis (Baxi et al., 2006; Diallo et al., 2011) between each triplex reaction and its three corresponding singular reactions. As shown in Table 3, correlation coefficients between each triplex reaction and its corresponding singular assays were from 0.972 to 0.999 when plasmid DNAs were used, and 0.975–0.998 when RNA were used, which indicates that there was no noticeable interference or reduced sensitivity observed by multiplexing the three targets into each subpanel.

3.4. Assay specificity

The specificity of the multiplex real-time RT-PCR panel with plasmid DNA was evaluated by comparing two pools of control plasmids: one has all standard control plasmids that were used in the whole panel (target pool); the other pool was made with the same pool of plasmids for the whole panel but without the targeted plasmid (non-target pool). The results showed that only the intended target gene was amplified from its target templates, and there was no signal detected in all non-target pools. The triplex real-time assay also correctly identified all of its three target genes from the mixed template and no cross-amplification was observed. Diagnostic specificity on 230 target samples were 100% as confirmed by other diagnostics (detailed in Section 3.5 below).

In addition, a total of 91 clinical samples positive for other non-target porcine viral pathogens were tested with the newly developed real-time PCR assay. We did not observe any non-specific positive signals in the 91 clinical samples.

3.5. Testing on clinical samples

Testing on a panel of 80 clinical serum samples revealed that 43 were positive for PRRSV-NA, one was positive for SIV, one was positive for PRRSV-EU, and five were dual-positive for PRRSV-NA and SIV. Testing of 53 lung tissues showed that 28 were positive for SIV, three were positive for PRRSV-NA and five tissues were co-infected with SIV and PRRSV-NA. Compared to the newly developed singular real-time RT-PCR assays, the triplex real-time RT-PCR detected not only the intended target viruses that were previously identified by other methods, but also the other co-infecting viruses that were not included in the previous detection methods (Table 4). All positive samples identified by previous methods also tested positive by our newly developed triplex real-time RT-PCR, except that the former assays didn't differentiate PRRSV-NA and PRRSV-EU (Table 4).

Table 4
Comparison among the newly developed triplex SIV, PRRSV-NA and PRRSV-EU real time RT-PCR assay with existing real time RT-PCR assays using clinical samples.

Clinical samples	Target	Newly developed real time RT-PCR				KSU real time RT-PCR	
		Triplex of SIV/PRRSV-NA/PRRSV-EU	SIV singular	PRRSV-NA singular	PRRSV-EU singular	PRRSV	SIV
80 sera	SIV	1	1	0	0	NT	NT
	PRRSV-NA	43	0	43	0	44	NT
	PRRSV-EU	1	0	0	1		
	SIV + PRRSV-NA	5	5	5	0	NT	NT
53 lung tissues	SIV	28	28	0	0	NT	28
	PRRSV-NA	3	0	3	0	NT	NT
	PRRSV-EU	0	0	0	0	NT	NT
	SIV + PRRSV-NA	5	5	5	0	NT	NT

NT: Not tested.

Table 5
Comparison among the newly developed triplex PPV, PRV and PCV-2 real time PCR assay with existing real time PCR assays using clinical samples.

Clinical samples	Positive target(s)	Newly developed real time PCR				BJCIQ real time PCR		
		Triplex of PPV, PRV and PCV-2	PPV singular	PRV singular	PCV-2 singular	PPV	PRV	PCV-2
24 tonsil tissues positive for PPV	PPV	24	24	0	0	24	NT	NT
	PRV	3	0	3	0	NT	NT	NT
	PCV-2	12	0	0	12	NT	NT	NT
	PPV + PRV	3	3	3	0	NT	NT	NT
	PPV + PCV-2	12	12	0	12	NT	NT	NT
	PRV + PCV-2	3	0	3	3	NT	NT	NT
	PPV + PRV + PCV-2	3	3	3	3	NT	NT	NT
24 tonsil tissues positive for PCV-2	PPV	10	10	0	0	NT	NT	NT
	PRV	4	0	4	0	NT	NT	NT
	PCV-2	24	0	0	24	NT	NT	24
	PPV + PRV	4	4	4	0	NT	NT	NT
	PPV + PCV-2	10	10	0	10	NT	NT	NT
	PRV + PCV-2	4	0	4	4	NT	NT	NT
	PPV + PRV + PCV-2	4	4	4	4	NT	NT	NT
16 lung tissues positive for PRV	PPV	8	8	0	0	NT	NT	NT
	PRV	14	0	14	0	NT	16	NT
	PCV-2	5	0	0	5	NT	NT	NT
	PPV + PRV	7	7	7	0	NT	NT	NT
	PPV + PCV-2	5	5	0	5	NT	NT	NT
	PRV + PCV-2	5	0	5	5	NT	NT	NT
	PPV + PRV + PCV-2	1	1	1	1	NT	NT	NT

NT: Not tested. BJCIQ: Beijing Entry & Exit Inspection and Quarantine Bureau.

Testing of clinical samples with the subpanel of triplex real-time PCR of PPV, PRV and PCV-2 showed that, among all of the 64 tissues diagnosed positive with the newly developed real-time PCR and virus isolation, 42 samples were positive for PPV, 41 positive for PCV-2 and 21 positive for PRV. There were 14 samples that were positive for co-infections with PPV and PRV; 27 samples were positive for co-infections with PPV and PCV-2; 12 samples were positive for co-infections with PRV and PCV-2; and 8 samples were co-infected with all three viruses (Table 5). The results were the same between the newly developed triplex RT-PCR assays and the routine real-time RT-PCR assays used in Animal Quarantine Lab of Beijing Entry & Exit Inspection and Quarantine Bureau (BJCIQ).

Fifteen lymph node samples that were tested positive for FMDV by both virus isolation and RT-PCR from Chinese National Foot and Mouth Disease Reference Laboratory (CNFMDRL) were included in the validation. An additional 14 lung tissue samples, of which all 14 tested CSFV positive by both virus isolation and RT-PCR at Chinese National Classical Swine Fever Reference Laboratory (CNCSFRL), were used as clinical samples for further validation. Four ASFV strains confirmed by FADDL were also used for validation. The new triplex real-time RT-PCR for FMDV, CSFV and ASFV generated similar results in detecting FMDV, CSFV and ASFV as the real-time RT-PCR procedure and virus isolation currently used at CNFMDRL, CNCSFRL and FADDL (Table 6). There was no co-infection observed in these 29 FMDV and CSFV samples.

4. Discussion

Mixed infections with different viruses are common in swine production systems and some of them can cause similar clinical signs, which makes it difficult to diagnose. Rapid, multi-targets and high-throughput diagnostic approaches are in demand for identification of syndromic pathogens (Giammarioli et al., 2008; Wernike et al., 2013b). Moreover, with the increasing growth of international trades, animal transport and human traveling, the risk of transboundary spreading of some diseases is significantly higher (Wernike et al., 2013a). To prevent the spread of transboundary diseases into large geographic areas with high density animal populations, especially after emerging and reemerging of these diseases, rapid diagnosis is of utmost importance (Wernike et al., 2013a).

Here, a panel of multiplex real-time assays for simultaneous detection and differentiation of 12 important viruses and viral serotypes, was developed and validated. The whole panel contains 4 subpanels with three molecular targets in each subpanel. It is flexible to use as the whole panel, or as subpanels in any combination.

Table 6
Comparison between the newly developed triplex FMDV, CSFV and ASFV real time RT-PCR with virus isolation and existing real time RT-PCR assay on clinical samples.

Clinical samples	Target	Newly developed real time RT-PCR triplex assay of FMDV/CSFV/ASFV	CNCSFVRL/CNFMDVRL/FADDL-USDA[a]					
			Real-time RT-PCR			Virus isolation		
			FMDV	ASFV	CSFV	FMDV	ASFV	CSFV
15 lymph nodes positive for FMDV	FMDV	15	15	NT[b]	NT	15	NT	NT
	CSFV	0	NT	NT	NT	NT	NT	NT
	ASFV	0	NT	NT	NT	NT	NT	NT
14 lung tissues positive for CSFV	FMDV	0	NT	NT	NT	NT	NT	NT
	CSFV	14	NT	NT	14	NT	NT	14
	ASFV	0	NT	NT	NT	NT	NT	NT
4 ASFV isolates	FMDV	0	NT	NT	NT	NT	NT	NT
	CSFV	0	NT	NT	NT	NT	NT	NT
	ASFV	4	NT	4	NT	NT	4	NT

[a] CNCSFRL: Chinese National Classical Swine Fever Reference Laboratory; CNFMDRL: Chinese National Foot and Mouth Disease Reference Laboratory; FADDL: Foreign Animal Disease Diagnostic Laboratory, NVSL, APHIS, USDA.
[b] NT: Not tested.

The multiplexing approach could also be valuable in detecting untargeted pathogens that are included in this comprehensive swine pathogen panel.

The newly developed panel of multiplex real-time assays offers a rapid, high-throughput, and reliable screening system for the 12 major viruses in swine. The results of specificity analysis indicated that no cross-amplification or non-specific amplification was observed. The sensitivity analysis showed that the limit of detection is 1–10 copies per reaction for DNA, and 4–16 copies for RNA templates (Table 2). Comparison between each triplex and its three individual singular assays showed that the mean Ct values were almost overlapping with correlation coefficients (R^2) ranging from 0.972 to 0.999 for DNA and 0.975–0.998 for RNA templates (Table 2), which indicates that no interference is caused by multiplexing. One of the main problems of multiplex PCR assay is potential interaction among oligonucleotides in the same reaction that can cause reduced amplification sensitivity (Wernike et al., 2013b). This type of interaction wasn't observed in our study, which may be benefited from the in silico analysis of all primers and probes in each reactions.

The clinical sample test revealed that the two genotypes of Northern American and European PRRSV can be distinguished by the new assay, in which one PRRSV-EU positive was found from PRRSV positive sera. One SIV positive was found from samples negative for PRRSV before, and three positive for PRRSV-NA were also found from tissues negative for SIV; Moreover, five sera and five tissues were detected positive for co-infection with SIV and PRRSV-NA, respectively (Table 5).

PPV, PCV-2 and PRV are very common pathogens in swine production systems and some strains don't cause visible clinical signs, but may affect pig growth, especially for piglets. The clinical sample testing showed that co-infection of two or three viruses are also very common. Our data suggested that co-infection rate of PPV and PRV was 21.88% (14/64); for PPV and PCV-2 was up to 42.19% (27/64); for PRV and PCV-2 was 18.75% (12/64) and co-infection for PPV, PCV-2 and PRV was 12.5% (8/64) (Table 6).

The clinical samples positive for FMDV, CSFV, and ASFV were all pre-identified by virus isolation and real-time RT-PCR, and our results are in 100% agreement with previous results (Table 6). These isolated strains represent different genotypes of FMDV, CSFV, and ASFV, indicating the new triplex assay can cover different genotypes.

In conclusion, the newly developed panel of multiplex detection system allows the simultaneous detection and differentiation of 12 important swine viral infections. This panel assay could be potentially used in routine swine disease surveillance and diagnostics.

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