Development of A One-Step Multiplex qRT-PCR Assay For Detection of African Swine Fever Virus, Classical Swine Fever Virus And Atypical Porcine Pestivirus

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Abstract

Background: African swine fever virus (ASFV), classical swine fever virus (CSFV) and atypical porcine pestivirus (APPV) have caused great economic losses to the swine industry in China. Since there exist co-infections of ASFV, CSFV and APPV in certain pig herds, it is necessary to accurately and differentially detect these pathogens in the fields. In this study, a one-step multiplex real-time quantitative reverse transcription-polymerase chain reaction (multiplex qRT-PCR) was developed for simultaneous and differential detection of ASFV, CSFV and APPV.

Results: The developed one-step multiplex qRT-PCR was able to specifically detect ASFV, CSFV and APPV, but could not amplify other viruses, including porcine circovirus type 2 (PCV2), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), foot-and-mouth disease virus (FMDV), porcine parvovirus (PPV), porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine rotavirus (PRoV), porcine deltacoronavirus (PDCoV), border disease virus (BDV), bovine viral diarrhea virus type 1 (BVDV-1), BVDV-2 and so on. The detection limit of the assay was 2.52×10^{1} copies/μL for ASFV, CSFV and APPV. Repeatability test using standard plasmids showed that the coefficients of variation of the intra- and inter-assay were less than 2 %. The detection of 509 clinical samples collected in Guangxi Province, Southern China from October 2018 to December 2020 showed that the positive rates of ASFV, CSFV and APPV were 45.58 %, 12.57 % and 3.54 %, respectively, while the co-infection rates of ASFV and CSFV, ASFV and APPV, CSFV and APPV were 4.91 %, 1.38 %, 0.98 %, respectively. Phylogenetic analysis based on the nucleotide sequences of partial ASFV p72 gene showed that all ASFV strains from Guangxi Province, Southern China belonged to genotype I and II.

Conclusion: A one-step multiplex qRT-PCR with high specificity, sensitivity, accuracy and repeatability was successfully developed for simultaneous and differential detection of ASFV, CSFV and APPV.

Background

African swine fever virus (ASFV) is an enveloped double-stranded DNA virus and the only member of genus Asfivirus in the family *Asfarvirifae* [1]. ASFV can cause African swine fever (ASF), a notifiable disease to the World Organization for Animal Health (OIE), which is characterized by high fever, extensive hemorrhage, pulmonary edema and intensive necrosis of lymphoid tissue, with high morbidity and mortality [2]. ASF was firstly emerged in Kenya in the 1920s, then spread to Europe in 1957, and further extended over the Caucasus region and into southern Russia in 2007 [3, 4], and recently introduced into China in August 2018 [5]. The disease spread rapidly over the country in short time and severely disrupted Chinese swine industry [6].

Classical swine fever virus (CSFV) is an enveloped single-stranded, positive-sense RNA virus that belongs to the Pestivirus genus of the *Flaviviridae* family [7]. CSFV can cause classical swine fever (CSF), another notifiable disease to the OIE, which is characterized by high fever, leukopenia, extensive hemorrhage, convulsion and constipation or diarrhea, with high morbidity and mortality [8]. CSF was first reported in

Ohio, USA in 1833, and nowadays, CSF is still prevalent in many countries in the world [9, 10]. Although Chinese C-strain vaccine of CSFV was developed in 1950s and has been widely using since then in the fields, CSF is still sporadic in many regions in China [11, 12].

Atypical porcine pestivirus (APPV), together with CSFV, border disease virus (BDV), bovine viral diarrhea virus 1 (BVDV-1) and BVDV-2, belongs to the Pestivirus genus of the *Flaviviridae* family [13]. It was first discovered in USA in 2015 [14], and then reported in many countries in America, Asia and Europe [13, 15]. APPV was demonstrated to be the causative agent of type A-II congenital tremor (CT) in newborn piglets, characterized by generalized body shaking with variable degrees of hypomyelination in brain and spinal cord [14, 16], which is similar to type A-I CT caused by CSFV [17].

ASFV, CSFV and APPV are still prevalent in many countries and cause huge economic losses to the swine industry worldwide. ASF and CSF show similar clinical symptoms and pathological changes in the fields, such as high fever, leukopenia, extensive hemorrhage, constipation or diarrhea, and high mortality [2, 8]. Type A-II CT caused by APPV shows similar clinical manifestations with type A-I CT caused by CSFV in newborn piglets [16, 17]. So, it is very difficult to distinguish these diseases in the fields sometimes. Furthermore, ASFV, CSFV and APPV are epidemic in many countries at the same time, and co-infection of ASFV, CSFV and/or APPV in pig herds can not be ruled out [18, 19]. Therefore, it is very important to differentially detect these pathogens in the laboratory. Currently, several differential polymerase chain reaction (PCR)/reverse transcription (RT)-PCR and real-time quantitative PCR (qPCR)/qRT-PCR assays have been developed for detection of ASFV [20, 21], CSFV [22, 23], APPV [24], ASFV/CSFV [25, 26] and ASFV/CSFV/APPV [18]. However, no qRT-PCR assay capable of simultaneous and differential detection of ASFV, CSFV and APPV has been reported until now. The objective of this study was to develop a specific, sensitive and reproducible one-step multiplex qRT-PCR for simultaneous and differential detection of ASFV, CSFV and APPV.

Results

Construction of standard plasmids

The target fragments of ASFV p72 gene, CSFV 5' untranslated region (UTR) and APPV 5'UTR were amplified by PCR/RT-PCR, purified and ligated to pMD18-T vector (TaKaRa, Dalian, China), and then transferred into *E. coli* DH5α competent cells. The positive clones were cultured and the plasmid constructs were extracted and determined their concentrations. As a result, the original concentrations of the three standard plasmids named as p-ASFV, p-CSFV and p-APPV were 2.65×10^{10} copies/μL, 2.52×10^{10} copies/μL, 3.02×10^{10} copies/μL, respectively, and were used as positive standard plasmids for optimization and sensitivity detection of the multiplex qRT-PCR.

Optimal parameters of the multiplex qRT-PCR

After optimization, the reaction conditions, including annealing temperature, concentrations of primers and probes and so on were acquired, and the parameters for the developed multiplex qRT-PCR were

selected as follows: 10 μL of 2× One Step RT-PCR Buffer III (TaKaRa, Dalian, China), 0.4 μL of Ex Taq HS (5 U/μL) (TaKaRa, Dalian, China), 0.4 μL of PrimeScript RT Enzyme Mix II (RNA/DNA) (TaKaRa, Dalian, China), 0.4 μL of each of ASFV, CSFV and APPV primers (20 pmol/μL), 0.5 μL of ASFV-p72-P (20 pmol/μL), 0.4 μL of CSFV-5'UTR-P (20 pmol/μL), 0.3 μL of APPV-5'UTR-P (20 pmol/μL), 2.0 μL of total DNA/RNA and distilled water to a total volume of 20 μL. The amplification parameters were as follows: reverse transcription at 42℃ for 5 min, and inactivation at 95℃ for 10 s; and then 40 cycles of denaturation at 95℃ for 5 s, annealing and extension at 59℃ for 34 s. The fluorescent signals were determined at the end of each cycle.

Standard curves of the multiplex qRT-PCR

To make the standard curves of the multiplex qRT-PCR, the standard plasmids of p-ASFV, p-CSFV and p-APPV were 10-fold serially diluted and mixed together for a final concentrations of each plasmid from 2.52×10^8 to 2.52×10^0 copies/μL per reaction. As a result, all the R^2 of ASFV, CSFV and APPV standard curves were over 0.999, showing good linear relationship between initial template concentration and the threshold cycle (Ct) values (Fig. 1).

Specificity of the multiplex qRT-PCR

To evaluate the specificity of the assay, the RNAs/DNAs of ASFV, CSFV, APPV, and 12 other viruses, including porcine circovirus type 2 (PCV2), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), foot-and-mouth disease virus (FMDV), porcine parvovirus (PPV), porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine rotavirus (PRoV), porcine deltacoronavirus (PDCoV), BVDV-1, BVDV-2 and BDV, were used as templates for the multiplex qRT-PCR. As a result, ASFV, CSFV and APPV showed specific amplification curves, while all 12 other viruses did not show any fluorescent signal and amplification curve, showing high specificity of the assay (Fig. 2).

Sensitivity of the multiplex qRT-PCR

The standard plasmids of p-ASFV, p-CSFV and p-APPV were 10-fold serially diluted from 2.52×10^8 to 2.52×10^0 copies/μL (final reaction concentrations: from 2.52×10^7 copies/μL to 2.52×10^{-1} copies/μL)and used to determine the sensitivity of the multiplex qRT-PCR. As a result, the detection limit of the multiplex qRT-PCR was 2.52×10^0 copies/μL for ASFV, CSFV and APPV per reaction (Fig. 3).

Repeatability of the multiplex qRT-PCR

To evaluate the repeatability of the assay, three concentrations of each standard plasmid in the mixtures with a final reaction concentrations of 2.52×10^7, 2.52×10^5 and 2.52×10^3 copies/μL were used as templates for intra- and inter-assay. The results showed that the coefficients of variation (CV) of the Ct values of intra- and inter-assay were less than 2 % (Table 2), indicating high repeatability of the assay.

Table 2 Repeatability analysis of the multiplex qRT-PCR

Plasmid	Concentration (copies/μL)	Ct values of intra-assay			Ct value of inter-asssay		
		X̄	SD	CV (%)	X̄	SD	CV (%)
p-ASFV	2.52×10^3	27.847	0.370	1.329	27.942	0.379	1.356
	2.52×10^5	20.738	0.168	0.810	20.747	0.207	0.998
	2.52×10^7	13.845	0.166	1.199	13.725	0.124	0.903
P-CSFV	2.52×10^3	28.001	0.153	0.546	27.946	0.186	0.666
	2.52×10^5	20.359	0.200	0.982	20.457	0.239	1.168
	2.52×10^7	13.109	0.156	1.190	13.076	0.088	0.673
P-APPV	2.52×10^3	27.328	0.152	0.556	27.314	0.137	0.502
	2.52×10^5	19.125	0.267	1.396	19.128	0.207	1.082
	2.52×10^7	12.348	0.145	1.174	12.302	0.157	1.276

Detection results of the clinical samples by the multiplex qRT-PCR

A total of 424 clinical samples collected in Guangxi Province, Southern China from October 2018 to December 2020 were detected by the developed multiplex qRT-PCR for evaluate its practicality in the fields. As a result, the positive rates of ASFV, CSFV and APPV were 45.58 % (232/509), 12.57 % (64/509) and 3.54 % (18/509), respectively, while the co-infection rates of ASFV and CSFV, ASFV and APPV, CSFV and APPV were 4.91 % (25/509), 1.38 % (7/509), 0.98 % (5/509), respectively (Table 3).

Table 3
Detection results of clinical samples by the multiplex qRT-PCR

Date	Numbers	ASFV (%)	CSFV (%)	APPV (%)	ASFV + CSFV (%)	ASFV + APPV (%)	CSFV + APPV (%)
Oct, 2018	18	0 (0)	2 (11.11)	1 (5.56)	0 (0)	0 (0)	0 (0)
Nov, 2018	40	0 (0)	9 (22.5)	4 (10.00)	0 (0)	0 (0)	1 (2.50)
Dec, 2018	30	5 (16.67)	6 (20.00)	1 (3.33)	3 (10.00)	0 (0)	1 (3.33)
Jan, 2019	38	5 (13.16)	4 (10.53)	0 (0)	1 (2.63)	0 (0)	0 (0)
Feb, 2019	57	15 (26.32)	5 (8.77)	3 (5.26)	3 (5.26)	2 (3.51)	1 (1.75)
Mar, 2019	36	10 (27.78)	7 (19.44)	0 (0)	4 (11.11)	0 (0)	0 (0)
Apr, 2019	19	12 (63.16)	3 (15.79)	1 (5.26)	0 (0)	0 (0)	0 (0)
May, 2019	16	16 (100.00)	0 (0)	2 (12.50)	0 (0)	1 (6.25)	0 (0)
Jun, 2019	11	4 (36.36)	1 (9.09)	1 (9.09)	0 (0)	0 (0)	0 (0)
Jul, 2019	12	12 (100.00)	2 (16.67)	0 (0)	0 (0)	0 (0)	0 (0)
Aug, 2019	28	20 (71.43)	2 (7.14)	0 (0)	2 (7.14)	0 (0)	0 (0)
Sep, 2019	15	15 (100.00)	2 (13.33)	0 (0)	2 (13.33)	0 (0)	0 (0)
Oct, 2019	20	20 (100.00)	1 (5.00)	0 (0)	1 (5.00)	0 (0)	0 (0)
Nov, 2019	24	21 (87.50)	5 (20.83)	3 (12.50)	5 (20.83)	3 (12.50)	2 (8.33)
Dec, 2019	10	6 (60.00)	1 (10.00)	0 (0)	0 (0)	0 (0)	0 (0)
Jan, 2020	10	10 (100.00)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Feb, 2020	10	7 (70.00)	1 (10.00)	0 (0)	0 (0)	0 (0)	0 (0)

Date	Numbers	ASFV (%)	CSFV (%)	APPV (%)	ASFV + CSFV (%)	ASFV + APPV (%)	CSFV + APPV (%)
Jul, 2020	4	0 (0)	4 (100.00)	0 (0)	0 (0)	0 (0)	0 (0)
Aug, 2020	10	8 (80.00)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Sep, 2020	26	14 (53.85)	2 (7.69)	0 (0)	0 (0)	0 (0)	0 (0)
Oct, 2020	20	13 (65.00)	6 (30.00)	0 (0)	4 (20.00)	0 (0)	0 (0)
Nov, 2020	32	15 (46.88)	1 (3.13)	0 (0)	0 (0)	0 (0)	0 (0)
Dec, 2020	23	4 (17.39)	0 (0)	2 (8.70)	0 (0)	0 (0)	0 (0)
Total	**509**	**232 (45.58)**	**64 (12.57)**	**18 (3.54)**	**25 (4.91)**	**7 (1.38)**	**5 (0.98)**

Phylogenetic analysis based on ASFV p72 gene

A total of 21 clinical samples were selected randomly from ASFV positive samples and partial p72 gene of ASFV were amplified and sequenced. Phylogenetic analysis based on partial p72 gene nucleotide sequences showed that 17 strains of the 21 strains obtained from Guangxi Province, together with strains from Mozambique (MOZ-60-98, MAD/1/98, MAZ 9/2006), Tanzania (TAN 2011/01) and Zambia (LUS93-1), formed a distinguishable cluster belonging to genotype II, and the other 4 strains, together with strains from Nigeria (Nig01, NIG-2), Angola (Ang72), Congo (Kat67), Ghana (GHA/1/00), South Africa (ZAR85) and Cameroon (CAM/4/85), belonged to genotype I (Fig. 4).

Discussion

ASFV and CSFV might co-infect in pig herds and their clinical manifestations and pathological changes might be hard to distinguish in the fields, so as CSFV and APPV [2, 8, 15, 16]. Since there exist co-infection and/or secondary infection of ASFV, CSFV and APPV in certain pig herds [18, 19], it is necessary to differentially detect these pathogens in the laboratory to accurately diagnose these diseases. The qRT-PCR is a rapid, specific, sensitive and accurate method for detection of viral nucleic acids, which could be conveniently used for quantification and detection of swine viral pathogens [27, 28]. Due to its high throughput, sensitivity, accuracy and ability to detect several pathogens with one reaction in very short time, multiplex qRT-PCR has been widely used in veterinary diagnostic laboratory in China. Therefore, a one-step multiplex qRT-PCR was developed in order to differentially detect ASFV, CSFV and APPV in this study. The assay could specifically detect ASFV, CSFV and APPV with a detection limit of 2.52×10^1 copies/μL for each pathogen, and the coefficients of variation of the intra- and inter-assay were all less than 2 %, showing high specificity, sensitivity and repeatability. Finally, the developed assay was used to detect 424 clinical samples to further verify its practicability in the fields.

The developed qRT-PCR was used to detect 509 clinical samples from Guangxi Province, Southern China for ASFV, CSFV and APPV. As a result, the positive rates of ASFV, CSFV and APPV were 45.58 %, 12.57 % and 3.54 %, respectively, indicating that ASFV, CSFV and APPV were widely popular in the pig herds in Southern China. Furthermore, it was noteworthy that the co-infection rates of ASFV/CSFV, ASFV/APPV and CSFV/APPV were 4.91 %, 1.38 % and 0.98 %, respectively, indicating that co-infections of ASFV, CSFV and APPV were common in pig herds to a certain extent. The results were similar to our previous report on the detection of ASFV, CSFV and APPV in the fields [19]. Since ASFV and CSFV co-infection could exacerbate the manifestations and pathological changes each other [18], and ASFV could suppress the immune response of pig herds which vaccinated with CSFV vaccine [29], it is very important to accurately detect the pathogens and rapidly eliminate the infected pigs in the early stage. Therefore, the developed multiplex qRT-PCR in this study could provide a useful tool for rapid differential detection of ASFV, CSFV and APPV in the fields.

ASF was firstly outbroke in China in August 2018, and then outbroke in other Asian countries [30], which caused huge economic losses to the swine industry. Since ASF was a newly emergent disease in China and has spread rapidly across this country [6], it is very interested to study the genomic characteristics of ASFV. The ASFV genome varies about 170 to 193 kb, which encodes for 150 to 167 proteins [31]. Based on partial p72 gene sequences, ASFV is currently classified into 24 genotypes and divided into three lineages [32], and 21 ASFV strains from Guangxi Province in this study shared a high level of nucleotide homology with 97.3 %~100 %, and shared 83.4 %~95.1 % nucleotide identity with strains from different countries in the world (data not showed). Phylogenetic analysis based on partial p72 gene sequences revealed that all ASFV strains from Guangxi Province belonged to two genotypes (genotype I and II), of which most strains were grouped into Genotype II and the other strains were grouped into genotype I. The results showed that genotype I and II ASFV strains were popular in Guangxi Province at the same time, which increased the complexity of pandemic strains and made it harder to prevent and control. Furthermore, genotype II ASFV strains may enable domestic pigs and wild boars to develop chronic infections and become carriers after recovery [30]. Since genotype II were the mainly popular strains in Guangxi Province, it is necessary to pay more attention to this phenomenon.

Conclusion

This is the first report on a one-step multiplex qRT-PCR, which indicated high sensitivity, specificity and repeatability, to provide an accurate tool for simultaneous and differential detection of ASFV, CSFV and APPV. The ASFV strains from Guangxi Province, Southern China belonged to genotype I and II.

Methods

Viruses and clinical samples

CSFV (C vaccine strain), PCV2 (SX07 vaccine strain), PRRSV (TJM-F92 vaccine strain), FMDV (O/Mya98/XJ/2010 vaccine strain), PRV (Bartha-K61 vaccine strain), PPV (N vaccine strain), PEDV

(CV777 vaccine strain), TGEV (H vaccine strain), PRoV (NX vaccine strain) were stored in our laboratory. The ASFV, APPV, BVDV-1, BVDV-2, BDV and PDCoV positive clinical samples were collected in the fields, confirmed by PCR/RT-PCR and gene sequencing, and stored in our laboratory.

A total of 509 clinical samples, including brain, lung, liver, spleen and lymph nodes for each naturally dead pig, were collected from different pig herds in Guangxi Province, Southern China from October 2018 to December 2020. All clinical samples were stored at -80°C until used.

Primers and TaqMan probes

Three pairs of specific primers and TaqMan probes used for multiplex qRT-PCR assay were designed using software Primer Express 3.0 basing on the genomic sequences of ASFV (GenBank accession number NC_001659), CSFV (NC_002657) and APPV (KY624591), respectively, which amplified 79 bp fragment for ASFV p72 gene, 72 bp fragment for CSFV 5'UTR and 90 bp fragment for APPV 5'UTR, respectively. The detailed information of primers and probes were listed in Table 1.

Table 1 Primers and probes used for detection of ASFV, CSFV and APPV

Primer and probe	Sequence (5'→3')	Product size (bp)
ASFV-p72-F	GGCGTATAAAAAGTCCAGGAAATTC	
ASFV-p72-R	TTCGGCGAGCGCTTTATC	79
ASFV-p72-P	Texas Red-TCACCAAATCCTTTTGCGATGCAAGCT-BHQ2	
CSFV-5'UTR-F	CCTGAGTACAGGACAGTCGTCAGT	
CSFV-5'UTR-R	CCCTCGTCCACATAGCATCTC	72
CSFV-5'UTR-P	JOE-TTCGACGTGAGCAGAAGCCCACC-BHQ1	
APPV-5'UTR-F	GGCGTGCCCAAAGAGAAAT	
APPV-5'UTR-R	GGCACTCTATCAAGCAGTAAGGTCTA	90
APPV-5'UTR-P	FAM-TCGGGTCCACCATGCCCCTTT-BHQ1	

Extraction of nucleic acid

All vaccine viruses and the pooled clinical tissue homogenates (20 %, W/V) were resuspended with phosphate buffer saline (PBS, pH7.2), vortexed and centrifuged at 12, 000× g at 4°C for 5 min. Total RNA and DNA was extracted from the supernatants using MiniBEST RNA/DNA Extraction Kit Ver.5.0 (TaKaRa, Dalian, China) according to the manufacture's instructions, and stored at -80°C until used.

Construction of standard plasmids

Total DNA was extracted from ASFV positive sample, and total RNA were extracted from CSFV vaccine and APPV positive sample and then reversed transcribed to cDNA. The target fragments of ASFV, CSFV and APPV were amplified by PCR using ASFV DNA and CSFV, APPV cDNA as templates. The amplicons were purified and cloned into pMD18-T vector (TaKaRa, Dalian, China) and transferred into *E. coli* DH5α competent cells (TaKaRa, Dalian, China). The positive clones were cultured at 37°C for 18 h-20 h and extracted by MiniBEST Plasmid Extraction Kit Ver.5.0 (TaKaRa, Dalian, China) for plasmid constructs. The plasmids were named as p-ASFV, p-CSFV and p-APPV, respectively, and stored at −80°C until used as standard plasmids.

The standard plasmids were quantified by ultraviolet absorbance at 260 nm and 280 nm with a NanoDrop Sectrophotometer (Thermo Fisher, USA). The exact copy numbers of plasmids were calculated using the following formula:

Plasmid copies/μL = $(6.02 \times 10^{23}) \times (X\ ng/\mu L \times 10^{-9})$/plasmid length (bp) × 660

Optimization of the single qRT-PCR assay

The standard plasmids were 10-fold serially diluted from 2.52×10^9 copies/μL to 2.52×10^1 copies/μL （final reaction concentrations: from 2.52×10^8 copies/μL to 2.52×10^0 copies/μL） for optimizing the reaction conditions of the single qRT-PCR of ASFV, CSFV and APPV. A total volume of 20 μL contained: 2× One Step qRT-PCR Buffer III (TaKaRa, Dalian, China) 10 μL, Ex Taq HS (5 U/μL) (TaKaRa, Dalian, China) 0.4 μL, PrimeScript RT Enzyme Mix II (TaKaRa, Dalian, China) 0.4 μL, each primer 0.1-0.6 μL, probe 0.1-0.6 μL, plasmid template 2.0 μL and distilled water to a total volume of 20 μL. All reactions were amplified by an ABI QuantStudio™ 6 Real-time System (ABI, USA) and the amplification parameters were as follows: 42℃ for 5 min, followed by 95℃ for 10 s; then 40 cycles of 95℃ for 5 s and 59℃ for 34 s. The fluorescent signals were determined at the end of each cycle.

Optimization of the multiplex qRT-PCR assay

Based on the optical reaction conditions of the single qRT-PCR, the reaction conditions of the multiplex qRT-PCR, imcluding annealing temperature, primer concentrations, probe concentrations and so on, were further determined by orthogonal experiments.

A total volume of 20 μL contained: 10 μL of 2× One Step qRT-PCR Buffer III (TaKaRa, Dalian, China), 0.4 μL of Ex Taq HS (5 U/μL) (TaKaRa, Dalian, China), 0.4 μL of PrimeScript RT Enzyme Mix II (TaKaRa, Dalian, China) , 0.1-0.6 μL of the mixtures of primers and probes with different final concentrations, 2.0 μL of the three standard plasmids mixed in ratio of 1:1:1 with different final concentrations as templates, and sterilized distilled water to a final volume of 20 μL. The amplification parameters were as follows: 42℃ for 5 min, followed by incubation at 95℃ for 10 s; then 40 cycles of denaturation at 95℃ for 5 s and annealing and extension at 59℃ for 34 s. Finally, the fluorescent signals were determined at the end of each cycle. After amplification, a Ct value was assigned to each sample. The final concentrations of

primers, probes and the amplification conditions were optimized to obtain the maximun ΔRn and minimal C_t using the standard plasmids of different dilutions as template.

Specificity analysis of the multiplex qRT-PCR

The DNA or RNA of ASFV, CSFV, APPV, PCV2, PRV, PRRSV, FMDV, PEDV, TGEV, PRoV, PDCoV, BVDV-1, BVDV-2 and BDV were used as templates of the developed multiplex qRT-PCR to verify the specificity of the assay.

Sensitivity analysis of the multiplex qRT-PCR

The standard plasmids of p-ASFV, p-CSFV and p-APPV were 10-fold serially diluted from 2.52×10^8 copies/μL to 2.52×10^0 copies/μL （final reaction concentrations: from 2.52×10^7 copies/μL to 2.52×10^1 copies/μL）and used as templates for the multiplex qRT-PCR to determine the sensitivity of the assay.

Repeatability analysis of the multiplex qRT-PCR

The standard plasmids of p-ASFV, p-CSFV and p-APPV were 10-fold serially diluted from 2.52×10^8 copies/μL to 2.52×10^0 copies/μL, and the concentrations of 2.52×10^8 copies/μL, 2.52×10^6 copies/μL and 2.52×10^4 copies/μL （final reaction concentrations: 2.52×10^7 copies/μL, 2.52×10^5 copies/μL and 2.52×10^3 copies/μL）were used as templates for the developed multiplex qRT-PCR. The coefficients of variation of the intra- and inter-assay were determined to evaluate the repeatability of the assay.

Detection of clinical samples by the multiplex qRT-PCR

A total of 509 clinical samples were collected from pig farms in Guangxi Province, Southern China from October 2018 to December 2020. The total RNA and DNA were extracted from 20 % tissue supernatants using MiniBEST RNA/DNA Extraction Kit Ver.5.0 (TaKaRa, Dalian, China) and were detected by the developed multiplex qRT-PCR for ASFV, CSFV and APPV.

Phylogenetic analysis based on ASFV p72 gene

Twenty-one samples were selected randomly from the positive samples of ASFV to amplify partial p72 gene using a pair of primers (P72-U: 5'-GGCACAAGTTCGGACATGT-3', P72-D: 5'-GTACTGTAACGCAGCACAG-3') as previously described [33]. The PCR products were purified, ligated to pMD-18T vector (TaKaRa, Dalian, China) and transferred to *E. coli.* DH5α competent cells. The positive clones were selected and sequenced (TaKaRa, Dalian, China), and the acquired sequences were edited by the EditSeq program of the DNAstar software, and aligned with the reference strains retrieved from GenBank using Clustal W. Phylogenetic reconstruction was conducted using the Maximum-Likelihood algorithm method (T92+G). Phylogenetic tree reliability was supported using the Kimura distances and a bootstrap method with 1000 replications.

Abbreviations

APPV: atypical porcine pestivirus; ASFV: African swine fever virus; BDV: border disease virus; BVDV: bovine viral diarrhea virus; CSFV: classical swine fever virus; CV: Coefficient of variation; FMDV: foot-and-mouth disease virus; multiplex qRT-PCR: multiplex real-time quantitative RT-PCR; OIE: The World Organization for Animal Health; PCV2: porcine circovirus type 2; PDCoV: porcine deltacoronavirus; PEDV: porcine epidemic diarrhea virus; PPV: porcine parvovirus; PRRSV: porcine reproductive and respiratory syndrome virus; PRV: pseudorabies virus; TGEV: transmissible gastroenteritis virus; PRoV: porcine rotavirus; RT-PCR: reverse-transcription polymerase chain reaction.

Declarations

Acknowledgements

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Authors' contributions

LHX carried out the experiments, data analysis and drafted the manuscript. SKC initiated the research program, and contributed to manuscript revision and final presentation. ZJ and CYT helped to perform the experiments. YYW and LWJ participated in sample collection. SHB revised the manuscript. QSJ and LF participated in clinical data acquisition. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article and are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The programs and procedures used in this study have been examined and approved by Guangxi CADC, China. We obtained written informed consent to use the clinical samples in our study from the owners of the animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

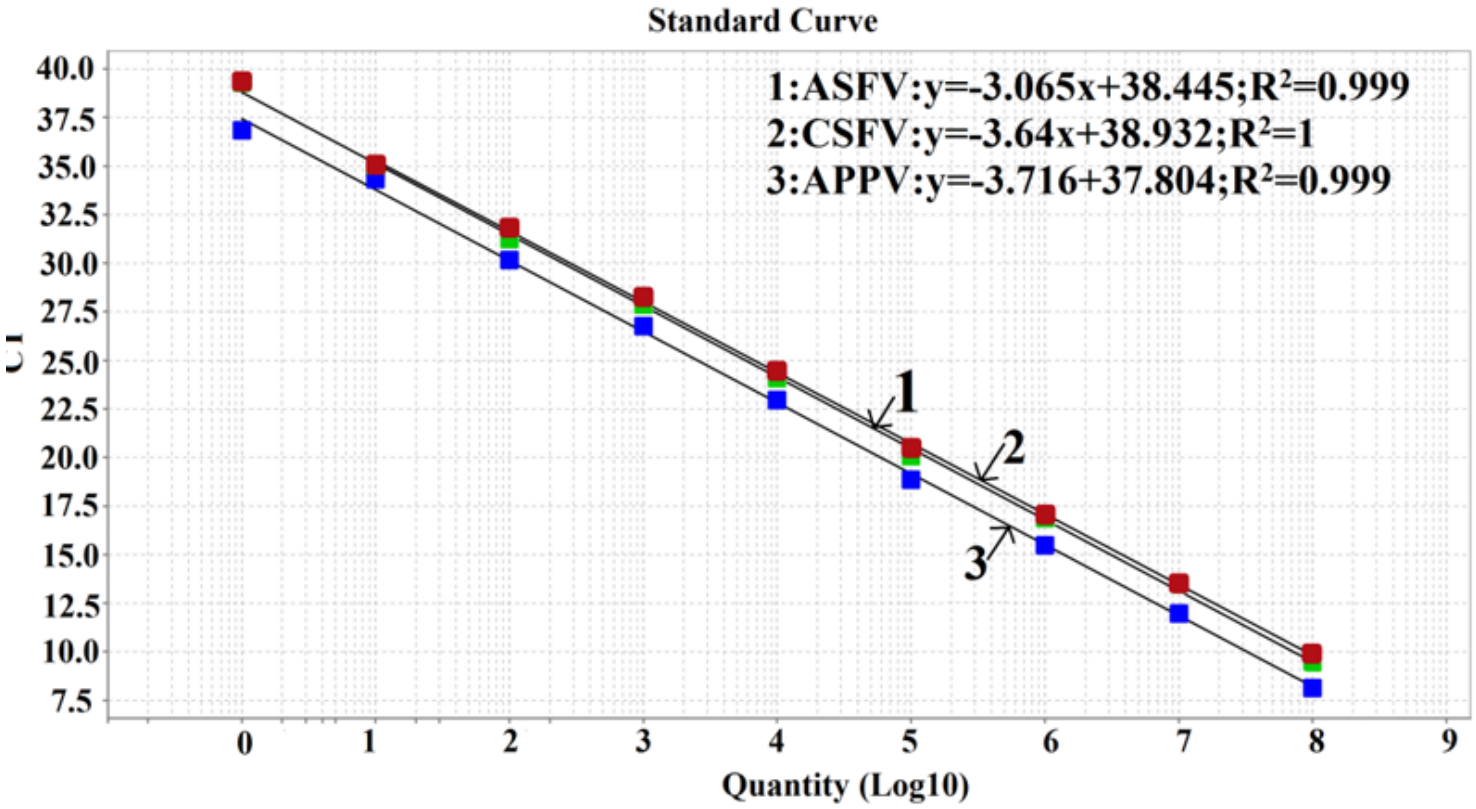

Figure 1

Standard curves of the multiplex qRT-PCR

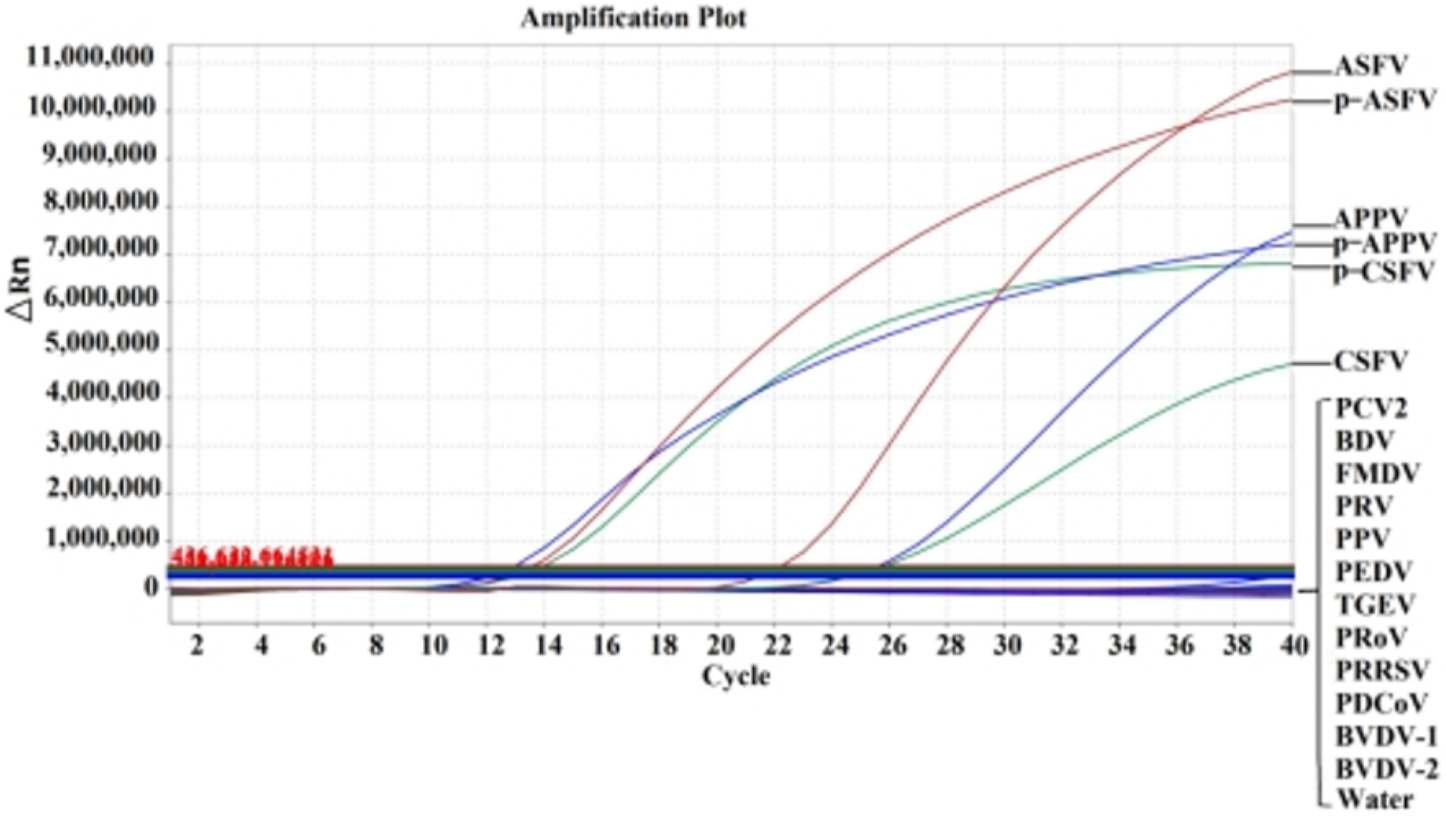

Figure 2

Specificity analysis of the multiplex qRT-PCR

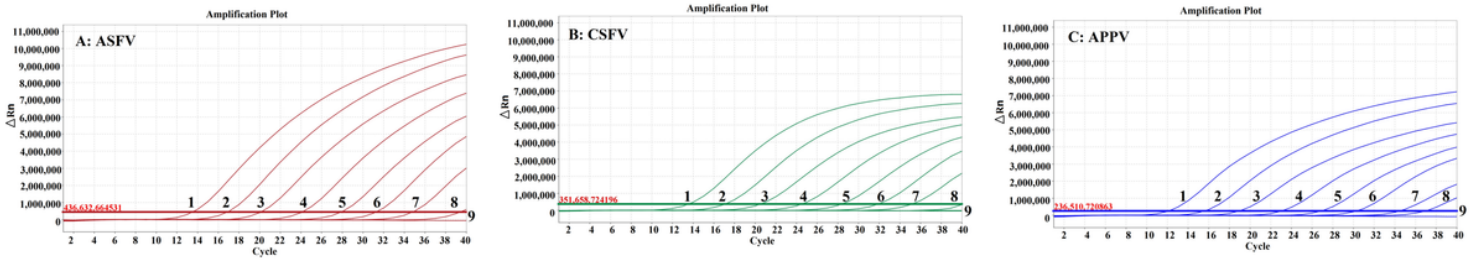

Figure 3

Sensitivity analysis of the multiplex qRT-PCR 1~9: 2.52 × 107 copies/μL~2.52 × 10-1 copies/μL (final reaction concentrations)

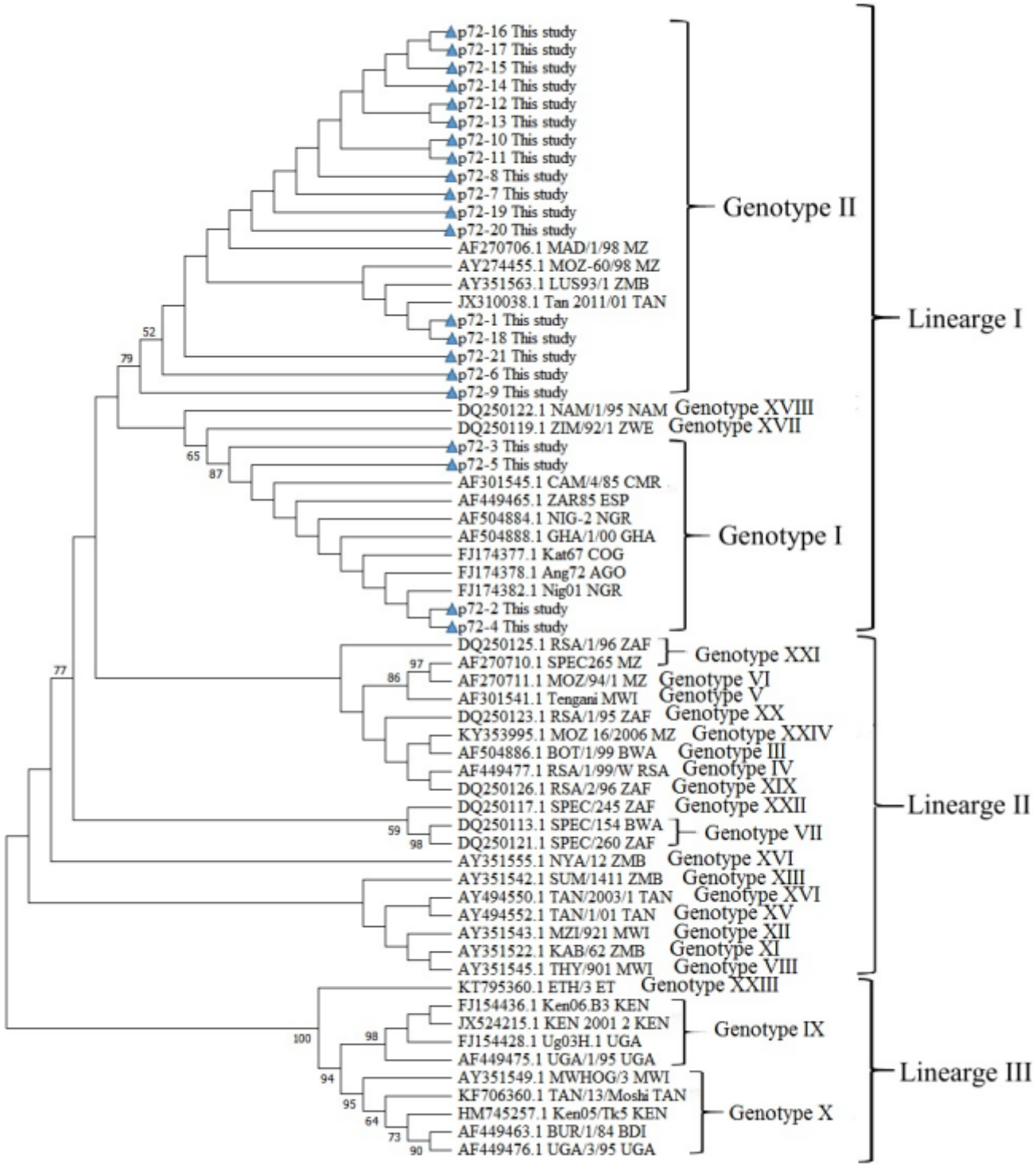

Figure 4

Phylogenetic tree based on partial p72 gene of ASFV