Development of a multiplex qRT-PCR assay for detection of African swine fever virus, classical swine fever virus and porcine reproductive and respiratory syndrome virus

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ABSTRACT

Background: African swine fever virus (ASFV), classical swine fever virus (CSFV), and porcine reproductive and respiratory syndrome virus (PRRSV) are still prevalent in many regions of China. Co-infections make it difficult to distinguish their clinical symptoms and pathological changes. Therefore, a rapid and specific method is needed for the differential detection of these pathogens.

Objectives: The aim of this study was to develop a multiplex real-time quantitative reverse transcription polymerase chain reaction (multiplex qRT-PCR) for the simultaneous differential detection of ASFV, CSFV, and PRRSV.

Methods: Three pairs of primers and TaqMan probes targeting the ASFV p72 gene, CSFV 5′ untranslated region, and PRRSV ORF7 gene were designed. After optimizing the reaction conditions, including the annealing temperature, primer concentration, and probe concentration, multiplex qRT-PCR for simultaneous and differential detection of ASFV, CSFV, and PRRSV was developed. Subsequently, 1,143 clinical samples were detected to verify the practicality of the assay.

Results: The multiplex qRT-PCR assay could specifically and simultaneously detect the ASFV, CSFV, and PRRSV with a detection limit of $1.78 \times 10^0$ copies for the ASFV, CSFV, and PRRSV, but could not amplify the other major porcine viruses, such as pseudorabies virus, porcine circovirus type 1 (PCV1), PCV2, PCV3, foot-and-mouth disease virus, porcine parvovirus, atypical porcine pestivirus, and Senecavirus A. The assay had good repeatability with coefficients of variation of intra- and inter-assay of less than 1.2%. Finally, the assay was used to detect 1,143 clinical samples to evaluate its practicality in the field. The positive rates of ASFV, CSFV, and PRRSV were 25.63%, 9.36%, and 17.50%, respectively. The co-infection rates of ASFV+CSFV, ASFV+PRRSV, CSFV+PRRSV, and ASFV+CSFV+PRRSV were 2.45%, 2.36%, 1.57%, and 0.17%, respectively.

Conclusions: The multiplex qRT-PCR developed in this study could provide a rapid, sensitive, specific diagnostic tool for the simultaneous and differential detection of ASFV, CSFV, and PRRSV.

Keywords: African swine fever virus; classical swine fever virus; porcine reproductive and respiratory syndrome virus; multiplex real-time quantitative RT-PCR (multiplex qRT-PCR)
INTRODUCTION

The African swine fever virus (ASFV), an enveloped double-stranded DNA virus and the only member of *Asfivirus* genus of the *Asfarviridae* family [1], can cause African swine fever (ASF), which is characterized by high fever, extensive hemorrhage, pulmonary edema, and intensive necrosis of lymphoid tissue with high morbidity and mortality [2]. The classical swine fever virus (CSFV), an enveloped single-stranded positive-sense RNA virus, belongs to the *Pestivirus* genus of the *Flaviviridae* family [3]. The virus can cause classical swine fever (CSF), which is characterized by high fever, leukopenia, extensive hemorrhage, convulsion, constipation, or diarrhea with high morbidity and mortality [4]. Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped single-stranded positive-sense RNA virus of the *Arterivirus* genus of the *Arteriviridae* family that can be divided into 2 genotypes: PRRSV1 (European genotype) and PRRSV2 (North American genotype) [5]. These viruses can cause porcine reproductive and respiratory syndrome (PRRS), which is characterized by mild to severe respiratory disease in infected newborn and growing pigs and reproductive failure in pregnant sows [6]. All ASF, CSF, and PRRS are notifiable diseases to the World Organization for Animal Health (OIE) and can cause severe economic losses to the swine industry and world trade of pork products. The 3 viruses can cause similar clinical symptoms and pathological changes, including high fever, extensive hemorrhage, respiratory and reproductive disorders, which makes it very difficult for differential diagnosis of these 3 diseases. Furthermore, co-infections with ASFV, CSFV, and PRRSV occur frequently in swine herds in China and elsewhere around the world [7-9]. In order to diagnose and deal with these diseases accurately and quickly, it is essential to develop a specific, sensitive, and rapid assay capable of the simultaneous and differential detection of ASFV, CSFV, and PRRSV.

Real-time quantitative polymerase chain reaction (PCR)/reverse transcription PCR (RT-PCR) is a sensitive, accurate, and rapid method for the detection and quantification of the target genome, which is based on continuous measurements of the accumulation or reduction of fluorescent signals during the amplification reaction [10]. Until now, most real-time assays are based on the use of target-specific TaqMan probes [11]. In particular, multiplex real-time quantitative PCR/RT-PCR (multiplex qPCR/RT-PCR) can detect several target genes with one reaction at one time. Therefore, it is used widely to diagnose and assess viral infectious diseases [12,13]. For ASFV, CSFV, and PRRSV detection in veterinary diagnosis, several multiplex qRT-PCR assays have been developed for detection of ASFV+CSFV [14,15], CSFV+PRRSV [16,17], and ASFV+CSFV+PRRSV [18]. On the other hand, none of the reports detected any ASFV positive sample in the field. Since the first outbreak of ASF in August, 2018 in China, it spread throughout the country in a very short time [19,20]. Currently, ASFV, CSFV, and PRRSV are simultaneously prevalent in many regions of China [21,22]. Hence, it is necessary to develop a specific and rapid method for the differential detection of these 3 viruses. The purpose of this study was to develop a TaqMan-based multiplex qRT-PCR for the simultaneous and differential detection of ASFV, CSFV, and PRRSV.

MATERIALS AND METHODS

**Viral strains**

ASFV (clinical positive samples), CSFV (C vaccine strain), PRRSV (TJM-F92 vaccine strain), porcine pseudorabies virus (PRV, Bartha-K61 vaccine strain), foot-and-mouth disease virus (FMDV, O/Mya98/XJ/2010 vaccine strain), porcine parvovirus (PPV, N vaccine strain),
porcine circovirus type 1 (PCV1, clinical positive samples), PCV2 (SX07 vaccine strain), PCV3 (clinical positive samples), atypical porcine pestivirus (APPV, clinical positive samples), and Senecavirus A (SVA, clinical positive samples) were stored in the laboratory.

Clinical samples
From February 2018 to March 2021, 1,143 clinical samples (including brain, lung, liver, spleen, kidney, tonsil, and lymph nodes of dead pigs) were collected from different pig farms in Guangxi Province, Southern China. Written informed consent was obtained to use the clinical samples in this study from the owners of the animals. Guangxi Center for Animal Disease Control and Prevention was approved by the Ministry of Agriculture and Rural Affairs of the People's Republic of China for the collection and detection of ASFV in clinical samples (approval No. 2018-154-25).

Design of primers and probes
Three pairs of specific primers and corresponding TaqMan probes were designed targeting the ASFV p72 gene, CSFV 5′ untranslated region (UTR), and PRRSV ORF7 gene, respectively. Table 1 lists detailed data relating to the primers and probes.

Nucleic acid extraction and reverse transcription
The total DNA and RNA were extracted from 200 μL of 20% (w/v in phosphate-buffered saline [PBS], pH 7.2) pooled tissue homogenates or a vaccine solution using a MiniBEST Viral RNA/DNA Extraction Kit Ver.5.0 (TaKaRa, China) and then reverse transcribed to complementary DNA (cDNA) using a PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer’s instructions. The extracted DNA and synthetic cDNA were stored at −20°C until used.

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

The standard plasmids were quantified by ultraviolet absorbance at 260 nm and 280 nm using a NanoDrop spectrophotometer (Thermo Fisher, USA). Their concentrations were calculated

| Table 1. Primers and probes for the detection of ASFV, CSFV, and PRRSV |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Names           | Sequences (5′→3′) | Product size (bp) |
| ASFV-p72-F      | GGCCTATAAAAAAGTCCAGGAAAATC | 79               |
| ASFV-p72-R      | TTTGGCCAGGGCTTATC | 79               |
| ASFV-p72-P      | FAM-TCACCAAATCTTTTGGATGCAAGCT-BHQ1 | 72               |
| CSFV-5′UTR-F    | CCTGAGTACAGGAGCGTGTCGTCG | 72               |
| CSFV-5′UTR-R    | CCCGTCGTCACATACATCTC | 72               |
| CSFV-5′UTR-P    | VIC-TTGACGTGAGCAGAGCACCACC-BHQ1 | 72               |
| PRRSV-ORF7-F    | GGTGTTGCTTCTGCTGAGCC | 178              |
| PRRSV-ORF7-R    | CGGACCCCAAGACGGAGG | 178              |
| PRRSV-ORF7-P    | Cy5-ATTCGTCCTCTGACCACCG-BHQ3 | 178              |

ASFV, African swine fever virus; CSFV, classical swine fever virus; PRRSV, porcine reproductive and respiratory syndrome virus; UTR, untranslated region.
as follows: plasmid copy number (copies/μL) = (plasmid concentration × 10^{-9} × 6.02 × 10^{23})/(660 \text{ Dalton/bases} × \text{DNA length}).

**Optimization of the reaction conditions of the multiplex qPCR**

The reaction conditions of the multiplex qPCR, including the annealing temperature, primer concentrations, and probe concentrations, were optimized using the QuantStudio 5 qPCR detection system (ABI, USA). The following basic systems were used to determine the optimal reaction conditions of the multiplex qPCR with a total volume of 25 μL: Premix Ex Taq (Probe qPCR) (TaKaRa) 12.5 μL, a mixture of 3 standard plasmids (with 10^7 copies of each plasmid) 2.5 μL, mixed 3 pairs of primers and 3 probes of different final concentrations and distilled water to a final volume of 25 μL. The amplification parameters were as follows: denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 56°C for 30 sec. The acquisition of fluorescent signals was recorded at the end of each cycle. The following parameters were acquired using an arrangement and combination test for reaction conditions: the annealing temperature were from 56°C to 61°C; the primer concentrations were 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 pmol/μL, respectively; the probe concentrations were 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 pmol/μL, respectively. The final concentrations of the primers, probes, and the amplification conditions were optimized to obtain the maximum ΔRn and minimal threshold cycle (C_T) using the standard plasmids of different dilutions as a template.

**Construction of standard curves**

After optimizing the reaction conditions, 3 standard plasmids of p-ASFV, p-CSFV, and p-PRRSV were 10-fold serially diluted from 1.78 × 10^8 copies/μL to 1.78 × 10^1 copies/μL and used as templates to construct the standard curves of the multiplex qPCR.

**Specificity analysis of the multiplex qPCR**

The specificity of the established assay was evaluated using the DNA or cDNA of ASFV, CSFV, PRRSV, PRV, PCV1, PCV2, PCV3, FMDV, PPV, APPV, and SVA as templates for amplification using the developed multiplex qPCR.

**Sensitivity analysis of the multiplex qPCR**

Three standard plasmids of p-ASFV, p-CSFV, and p-PRRSV were serially diluted 10-fold and mixed at a 1:1:1 ratio, and the final concentration for each plasmid ranged from 1.78 × 10^{8} copies/μL to 1.78 × 10^{3} copies/μL. These diluted plasmids were used as templates to determine the detection limit of the multiplex qPCR.

**Repeatability analysis of the multiplex qPCR**

The intra-assay and inter-assay tests were performed to evaluate the repeatability of the multiplex qPCR. Three standard plasmids of p-ASFV, p-CSFV, and p-PRRSV were serially diluted 10-fold and mixed; 1.78 × 10^{8}, 1.78 × 10^{7}, 1.78 × 10^{6} copies/μL of the abovementioned plasmids were used as templates. All the reactions were run in triplicate.

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

A total of 1,143 clinical samples collected in Guangxi Province, Southern China, from February 2018 to March 2021, were detected using the developed multiplex qRT-PCR.

The tissue samples, including brain, lung, liver, spleen, and lymph nodes, were homogenized in a PBS solution (pH7.2) using a blender (QIAGEN, Germany) followed by shaking with small glass beads for 5 min. The homogenized material (10%, w/v) was then freeze-thawed 3
times and centrifuged at $10,000 \times g$ for 10 min at 4°C, and the supernatant was used to extract the total nucleic acid (DNA and RNA). The total nucleic acids were extracted from 200 μL of the supernatant using a MiniBEST RNA/DNA Extraction Kit (TaKaRa) according to the manufacturer’s protocol. Finally, the extracted nucleic acids were used directly to detect the ASFV, CSFV, and PRRSV by multiplex qRT-PCR established in this study and the conventional multiplex RT-PCR (mRT-PCR) established by Hu et al. [9] with some modification.

The main steps of the multiplex qRT-PCR were as follows. The multiplex qRT-PCR was carried out using the QuantStudio 5 quantitative real-time PCR detection system (ABI) with a total volume of 25 μL reaction system, including the following: 12.5 μL of 2× One-Step RT-PCR Buffer, 0.5 μL of Ex Taq HS (TaKaRa), 0.5 μL of PrimerScript RT Enzyme Mix, ASFV primers (forward and reward primer) (25 pmol/μL) 0.4 μL each, ASFV probe (25 pmol/μL) 0.4 μL, CSFV primers (forward and reward primer) (25 pmol/μL) 0.4 μL each, CSFV probe (25 pmol/μL) 0.5 μL, PRRSV primers (forward and reward primer) (25 pmol/μL) 0.3 μL each, PRRSV probe (25 pmol/μL) 0.3 μL, 2.5 μL of the total nucleic acids (DNA/RNA) as a template and distilled water to a final volume of 25 μL. The amplification parameters were as follows: reverse transcription at 42°C for 5 min, pre-denaturation at 95°C for 10 sec, 40 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 56°C for 34 sec. The fluorescent signals were determined at the end of each cycle.

The main steps of the conventional mRT-PCR were as follows. mRT-PCR was carried out using a PowerCycler Gradient detection system (Analytic Jena, Germany) with a total volume of 20 μL, including 10 μL of Taq PCR Master Mix (TaKaRa), 0.4 μL of forward and reverse primers (25 pmol/μL) for each virus (ASFV, CSFV, and PRRSV), 2.5 μL of total nucleic acids (DNA/RNA) as the template and distilled water to a final volume of 20 μL. The amplification parameters were as follows: Pre-denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 1 min; a final extension at 72°C for 10 min. The amplified products were electrophoresed on a 1.2% agarose gel and visualized using a UV transilluminator (UVI FineReader, France).

RESULTS

Construction of standard plasmids

The target fragments of the ASFV p72 gene, CSFV 5′UTR, and PRRSV ORF7 gene were amplified by RT-PCR/PCR, purified, and ligated to pMD18-T vector, and then transferred to E. coli DH5α competent cells. The positive clones were cultured, and the plasmid constructs were extracted and their concentrations were determined. As a result, the original concentrations of the standard plasmids called p-ASFV, p-CSFV, and p-PRRSV were $1.78 \times 10^{10}$ copies/μL, $2.08 \times 10^{10}$ copies/μL, and $2.15 \times 10^{10}$ copies/μL, respectively. These were used as the standard positive controls to optimize the reaction conditions of the multiplex qPCR.

Optimal reaction conditions of the multiplex qPCR

The standard plasmids carrying the target fragments were used as templates to optimize the reaction conditions of the multiplex qPCR. Based on orthogonal experiments, the optimal annealing temperature and the concentrations of primers and probes were acquired.

After optimization, the developed multiplex qRT-PCR with a total volume of 25 μL contained the following: 2× One-Step RT-PCR Buffer, 0.5 μL of Ex Taq HS (TaKaRa), 0.5 μL of
PrimerScript RT Enzyme Mix, ASFV primers (forward and reward primer) (25 pmol/μL) 0.4 μL each, ASFV probe (25 pmol/μL) 0.4 μL, CSFV primers (forward and reward primer) (25 pmol/μL) 0.5 μL, PRRSV primers (forward and reward primer) (25 pmol/μL) 0.3 μL each, PRRSV probe (25 pmol/μL) 0.3 μL, 2.5 μL of total DNA/RNA as a template and distilled water to a final volume of 25 μL. The amplification parameters were as follows: reverse transcription at 42°C for 5 min, pre-denaturation at 95°C for 10 sec; then 40 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 56°C for 34 sec, and the fluorescent signals were measured at the end of each cycle. The criteria for judging the outcome were as follows. A Ct value ≤ 35 cycles was considered positive, and a Ct value > 35 cycles was considered negative.

**Standard curves of the multiplex qPCR**
The standard curves of the multiplex qPCR were constructed by a 10-fold serial dilution of the standard plasmids ranging from $1.78 \times 10^8$ copies/μL to $1.78 \times 10^1$ copies/μL as templates. As a result, the corresponding slope of the equation, correlation coefficient ($R^2$), and amplification efficiency ($E$) were as follows: $-3.185$, $1.00$, and $106\%$, respectively, for ASFV; $-3.337$, $0.999$, and $99.4\%$, respectively, for CSFV; $-3.255$, $0.999$ and $102.9\%$, respectively, for PRRSV (Fig. 1), indicating an excellent linear relationship between the initial templates and Ct values.

**Fig. 1.** Dynamic curves (A-C) and standard curves (D) of multiplex real-time quantitative polymerase chain reaction.

1–8, $1.78 \times 10^9$ copies/μL– $1.78 \times 10^1$ copies/μL; ASFV, African swine fever virus; CSFV, classical swine fever virus; PRRSV, porcine reproductive and respiratory syndrome virus.
Specificity of the multiplex qPCR

The DNA or cDNA of different porcine viruses were used as templates of the developed multiplex qPCR. As a result, only ASFV, CSFV, and PRRSV showed amplification curves. The other viruses, including PRV, PCV1, PCV2, PCV3, FMDV, PPV, APPV, and SVA, did not show any fluorescent signals and amplification curves (Fig. 2).

Sensitivity of the multiplex qPCR

Three standard plasmids were serially diluted 10-fold from $1.78 \times 10^8$ copies/μL to $1.78 \times 10^2$ copies/μL to determine the detection limits of the multiplex qPCR. The detection limits of all p-ASFV, p-CSFV, and p-PRRSV were $1.78 \times 10^0$ copies/μL (Fig. 3), highlighting the very high sensitivity of the multiplex qPCR.

Repeatability of the multiplex qPCR

Three concentrations of $1.78 \times 10^6$, $1.78 \times 10^4$, and $1.78 \times 10^2$ copies/μL of standard plasmids were used to evaluate the reproducibility of the developed multiplex qPCR. As a result, the coefficients of variation of the intra- and inter-assay were all less than 1.2% (Table 2), indicating excellent repeatability of the assay.

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Table 2. Repeatability analysis of multiplex real-time quantitative polymerase chain reaction

| Plasmid | Concentration (copies/μL) | Ct values of intra-assay | Ct values of inter-assay |
|---------|---------------------------|-------------------------|-------------------------|
|         |                           | $\bar{X}$ | SD | CV (%) | $\bar{X}$ | SD | CV (%) |
| p-ASFV  | $1.78 \times 10^7$        | 29.94  | 0.09 | 0.30  | 29.90  | 0.21 | 0.70  |
|         | $1.78 \times 10^4$        | 23.13  | 0.04 | 0.17  | 23.03  | 0.12 | 0.52  |
|         | $1.78 \times 10^2$        | 17.42  | 0.09 | 0.52  | 17.33  | 0.17 | 0.98  |
| p-CSFV  | $1.78 \times 10^7$        | 29.78  | 0.08 | 0.27  | 30.18  | 0.34 | 1.13  |
|         | $1.78 \times 10^4$        | 23.71  | 0.20 | 0.84  | 23.53  | 0.24 | 1.02  |
|         | $1.78 \times 10^2$        | 17.17  | 0.07 | 0.41  | 17.94  | 0.08 | 0.46  |
| p-PRRSV | $1.78 \times 10^7$        | 30.13  | 0.28 | 0.93  | 30.38  | 0.34 | 1.12  |
|         | $1.78 \times 10^4$        | 23.61  | 0.18 | 0.76  | 23.52  | 0.09 | 0.38  |
|         | $1.78 \times 10^2$        | 17.72  | 0.13 | 0.73  | 17.59  | 0.12 | 0.68  |

SD, standard deviation; CV, coefficients of variation; ASFV, African swine fever virus; CSFV, classical swine fever virus; PRRSV, porcine reproductive and respiratory syndrome virus.
The detection results of clinical samples by the multiplex qRT-PCR
A total of 1,143 clinical samples were collected from February 2018 to March 2021 in Guangxi Province, Southern China, and detected using the developed multiplex qRT-PCR to validate its usability. As a result (Table 3), the positive rates of ASFV, CSFV, and PRRSV were 25.63% (293/1,143), 9.36% (107/1,143), and 17.50% (200/1,143), respectively. The positive rates of co-infections with ASFV+CSFV, ASFV+PRRSV, CSFV+PRRSV, and ASFV+CSFV+PRRSV were 2.45% (28/1,143), 2.36% (27/1,143), 1.57% (18/1,143), and 0.17% (2/1,143), respectively.

At the same time, all the 1,143 clinical samples were detected using the conventional mRT-PCR reported by Hu et al. [9] with some modification. The results showed that the positive rates of ASFV, CSFV, and PRRSV were 23.27%, 8.75%, and 17.15%, respectively, and the coincidence rate of the developed multiplex qRT-PCR and the reported mRT-PCR was 98.51%, 99.39%, and 99.65%, respectively (Table 4).

DISCUSSION

ASF, CSF, and PRRS are all highly contagious diseases that have seriously damaged the swine industry worldwide. These diseases are still prevalent in many countries, and co-infections with 2 or 3 pathogens of ASFV, CSFV, and PRRSV are common in some pig...
herds [21,22]. Because they sometimes show similar clinical symptoms and pathological changes, it is difficult to identify the actual causative agents depending only on clinical information [23,24]. Thus, it is essential to develop a reliable method for the differential detection of ASFV, CSFV, and PRRSV in the laboratory and accurately diagnose these 3 diseases in the field. The multiplex qPCR/RT-PCR uses several specific primers and probes to amplify several target genes in a single reaction. The assay is rapid, sensitive, accurate, high-throughput, and time-saving. Therefore, it is used widely to detect viruses in veterinary laboratories [11,12]. In this study, 3 pairs of specific primers and corresponding probes were designed. A multiplex TaqMan probe-based qRT-PCR was developed for the simultaneous and differential detection of ASFV, CSFV, and PRRSV. The assay showed a detection limit

### Table 3. Detection results of clinical samples by multiplex real-time quantitative reverse transcription polymerase chain reaction

| Data | Number | Number of positive samples | ASFV | CSFV | PRRSV | ASFV+CSFV | ASFV+PRRSV | CSFV+PRRSV | ASFV+CSFV+PRRSV |
|------|--------|-----------------------------|------|------|-------|-----------|-----------|-----------|------------------|
| Feb, 2018 | 10 | 0 | 2 | 4 | 0 | 0 | 1 | 0 |
| Mar, 2018 | 14 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| Apr, 2018 | 7 | 0 | 1 | 2 | 0 | 0 | 0 | 0 |
| May, 2018 | 11 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Jun, 2018 | 104 | 0 | 4 | 17 | 0 | 0 | 0 | 0 |
| Jul, 2018 | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| Aug, 2018 | 20 | 1 | 6 | 11 | 0 | 0 | 0 | 0 |
| Sep, 2018 | 53 | 0 | 3 | 12 | 0 | 0 | 0 | 0 |
| Oct, 2018 | 125 | 1 | 4 | 18 | 0 | 0 | 0 | 0 |
| Nov, 2018 | 107 | 1 | 14 | 28 | 1 | 0 | 1 | 0 |
| Dec, 2018 | 66 | 6 | 8 | 31 | 3 | 1 | 5 | 1 |
| Jan, 2019 | 38 | 5 | 4 | 3 | 1 | 0 | 1 | 0 |
| Feb, 2019 | 136 | 36 | 16 | 4 | 3 | 0 | 0 | 0 |
| Mar, 2019 | 36 | 10 | 7 | 5 | 4 | 0 | 1 | 0 |
| Apr, 2019 | 19 | 12 | 3 | 9 | 0 | 0 | 0 | 0 |
| May, 2019 | 16 | 16 | 0 | 0 | 0 | 0 | 0 | 0 |
| Jun, 2019 | 13 | 13 | 2 | 1 | 2 | 1 | 0 | 0 |
| Jul, 2019 | 24 | 21 | 5 | 1 | 5 | 0 | 0 | 0 |
| Aug, 2019 | 28 | 20 | 2 | 0 | 2 | 0 | 0 | 0 |
| Sep, 2019 | 15 | 15 | 2 | 0 | 2 | 0 | 0 | 0 |
| Oct, 2019 | 32 | 32 | 1 | 4 | 1 | 4 | 0 | 0 |
| Nov, 2019 | 34 | 21 | 9 | 12 | 0 | 10 | 1 | 0 |
| Dec, 2019 | 10 | 6 | 1 | 0 | 0 | 0 | 0 | 0 |
| Jan, 2020 | 10 | 9 | 0 | 10 | 0 | 9 | 0 | 0 |
| Jun, 2020 | 4 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Jul, 2020 | 4 | 0 | 4 | 0 | 0 | 0 | 0 | 0 |
| Sep, 2020 | 56 | 14 | 1 | 3 | 0 | 0 | 1 | 0 |
| Nov, 2020 | 32 | 15 | 1 | 0 | 0 | 0 | 0 | 0 |
| Dec, 2020 | 71 | 25 | 6 | 14 | 4 | 2 | 1 | 1 |
| Jan, 2021 | 22 | 12 | 0 | 2 | 0 | 0 | 0 | 0 |
| Mar, 2021 | 24 | 2 | 0 | 1 | 0 | 0 | 0 | 0 |
| Total | 1,143 | 293 | 107 | 200 | 28 | 27 | 18 | 2 |

Positive rate (%) 25.63 9.36 17.5 2.45 2.36 1.57 0.17

ASFV, African swine fever virus; CSFV, classical swine fever virus; PRRSV, porcine reproductive and respiratory syndrome virus.

* Co-infection with ASFV and CSFV; † Co-infection with ASFV and PRRSV; ‡ Co-infection with CSFV and PRRSV; § Co-infection with ASFV, CSFV, and PRRSV.

### Table 4. Agreements between multiplex qRT-PCR and conventional mRT-PCR

| Detection method | Number of positive samples | ASFV | CSFV | PRRSV |
|------------------|-----------------------------|------|------|-------|
| Multiplex qRT-PCR | 293/1,143 | 107/1,143 | 200/1,143 |
| Conventional mRT-PCR | 276/1,143 | 100/1,143 | 196/1,143 |
| Agreements | 98.51% | 99.39% | 99.65% |

qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction; mRT-PCR, multiplex reverse transcription polymerase chain reaction; ASFV, African swine fever virus; CSFV, classical swine fever virus; PRRSV, porcine reproductive and respiratory syndrome virus.
of 1.78 × 10^6 copies/μL, and could specifically detect ASFV, CSFV, and PRRSV without a cross-reaction with other porcine viruses. The assay was further used to detect 1,143 clinical samples to verify its practicality and usefulness in the field. At the same time, all the 1,143 clinical samples were detected using the conventional mRT-PCR assay reported by Hu et al. [9] with some modification, which indicated that the coincidence rate of the developed multiplex qRT-PCR and the reported mRT-PCR was more than 98%. The multiplex qRT-PCR was more sensitive than mRT-PCR.

The developed multiplex qRT-PCR was used to detect 1,143 clinical samples; 293 (25.63%), 107 (9.36%), and 200 (17.50%) samples were positive for ASFV, CSFV, and PRRSV, respectively. This suggests that ASF, CSF, and PRRS were still prevalent in Guangxi Province, Southern China. The first outbreak of ASF was in August, 2018 in China, and it then spread over the country. Currently, it is a common infectious disease in pig herds [25,26]. As for CSF, even though the live attenuated C-strain vaccine with good immune efficacy has been widely used in the field since the 1950s, CSF has not been wholly controlled since it is still sporadic or endemic in some regions of China [27,28]. The first outbreak of PRRS in China was in 1996 and has been prevalent since then [29]. The North American genotype PRRSV (including classical strains, highly pathogenic strains, and NADCO-like variant strains) and European genotype PRRSV are prevalent in China [30,31]. The diversity and variability of epidemic strains weaken the immune effect of PRRSV vaccines, resulting in PRRS in many regions in China [31,32]. Furthermore, there are co-infections with 2 or 3 pathogens of ASFV, CSFV, and PRRSV, which increase the damage to the immune systems, decrease the immune efficacy of vaccination, and exacerbate these 3 diseases [8,33,34]. The detection results of clinical samples from 2018 to 2021 showed that ASFV, CSFV, and PRRSV were still prevalent in Guangxi Province, Southern China, and there are co-infections of these viruses with 2.45% of ASFV+CSFV, 2.36% of ASFV+PRRSV, 1.57% of CSFV+PRRSV and 0.17% ASFV+CSFV+PRRSV, respectively. To the best of the authors’ knowledge, this is the first report detecting ASFV positive samples in the field by a multiplex qRT-PCR. Therefore, the developed multiplex qRT-PCR could provide a useful tool for the simultaneous differential detection of the ASFV, CSFV, and PRRSV.

In conclusion, multiplex qRT-PCR was developed for simultaneous and differential detection of ASFV, CSFV, and PRRSV to rapidly and accurately detect ASFV, CSFV, and PRRSV from clinical samples, providing a more convenient tool for the accurate diagnosis and epidemiological investigation of these diseases.

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