**ABSTRACT**

It is difficult to differentiate pigs that have been injected attenuated vaccines from those that are infected by a virulent virus. In this study, a duplex reverse transcription polymerase chain reaction (RT-PCR) was developed for simultaneous detection and identification of the pathogenic strain (Shimen-strain) and attenuated vaccine strain (C-strain) of classical swine fever virus (CSFV). A fragment of 438 bp or 265 bp was amplified from the genomic RNA of the Shimen-strain or the C-strain, respectively. Both types of viruses were simultaneously identified from the mixed samples of Shimen-strain and C-strain, while no PCR bands were obtained from the templates of some other porcine original viruses with the designed primers. The lowest limit of detection was 0.0616 pg for the C-strain RNA and 0.0844 pg for the Shimen-strain RNA. The developed RT-PCR was adopted to test CSFV from 65 collected samples. Compared with quantitative real-time PCR, the results indicated that the developed RT-PCR can not only be used to distinguish the virulent and attenuated CSFV, but also possesses high sensitivity for detecting CSFV. This is a convenient method to detect and differentiate pigs infected with virulent CSFV from the pigs immunized with the C-strain.

**KEYWORDS**

CSFV; duplex RT-PCR; detection; differential diagnosis

**Introduction**

Classical swine fever virus (CSFV) can spread via vertical and horizontal transmission directly, and via pork products and contaminated fomites indirectly [1]. Thus, strict control measures for CSFV should be applied to the suspicious herds that may have been infected [2]. It is effective to control classical swine fever (CSF) with attenuated vaccines [3] and some vaccines against CSF have been developed [4]. The attenuated lapinized vaccine strains are widely used around the world [5]. The hog cholera lapinized virus (HCLV strain) has been maintained in many countries for its good efficacy and safety, and was named 'Chinese vaccine strain (C-strain)' [6].

The standard CSFV reference virulent strain in China named Shimen was isolated in 1945 and the HCLV strain was developed in 1954, derived by serial passages of a virulent virus through rabbits [6]. A phylogenetic tree shows that Shimen and HCLV cluster into the same subgroup [7]. It is difficult to differentiate the animals infected with the wild-type virus from HCLV vaccinated pigs by serology [8,9]. Therefore, it is of particular importance to develop a sensitive and specific approach for distinguishing the virulent CSFV from the vaccine virus.

Many studies in this area are based on the different nucleotide sequences of the viruses. There is a notable insertion of 12–13 continuous T-rich nucleotide sequence in the 3′-nontranslated region (3′-NTR) of the attenuated CSFV genome [10,11]. However, this is not an optimal location for primer design for the 3′-NTR is difficult to reverse into cDNA. The real-time polymerase chain reaction (PCR) assay is faster and more sensitive than conventional reverse transcription PCR (RT-PCR) [12–17]. However, the expensive instruments and reagents make it difficult to apply widely [18]. Here, we compared the genomic sequences of epidemic CSFV and attenuated vaccine strains to identify different sequences between virulent strains and the C-strain. Two pairs of specific primers were designed for developing a duplex RT-PCR, in the hope that the simple duplex RT-PCR can become a convenient approach in identifying the CSFV-infected pigs within non-vaccinated and/or vaccinated swine herds.

**Materials and methods**

**Viruses and cells**

Virulent Shimen strain (F114) was obtained from China Institute of Veterinary Drug Control (Beijing). A virulent
CSFV isolate from pathological tissue was maintained in our lab. C-strain (HCLV) was purchased from China Animal Husbandry Group (Beijing). PRRSV (porcine reproductive and respiratory syndrome virus), PCV2 (porcine circovirus type 2), PPV (porcine parvovirus), PRV (porcine pseudorabies virus) and TGEV (transmissible gastroenteritis virus of swine) were obtained from the Laboratory of Veterinary Prevention (Yangling, Shaanxi). Swine umbilical vascular endothelial cells (SUVECs), porcine kidney epithelial (PK-15) cells and swine testicular (ST) cells were cultured in high-glucose Dulbecco’s Modified Eagle Medium (DMEM; GIBCO, Carlsbad, UK) containing 10% fetal bovine serum (FBS; GIBCO, UK) and 50 μg/mL heparin (Sigma–Aldrich, Merto KGaA, Darmstadt, Germany) at 37 °C with 5% CO2. SUVEC is an immortalized porcine cell line established and maintained at our lab. PK-15 and ST cells were gifts from Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

### Primers

The genomic sequences of 25 CSFV isolates (20 virulent strains and 5 attenuated vaccine strains) were aligned using MegAlign of DNAStar 7.0, including the Shimen strain (Accession No. AF092448, AF333000), C-strain (accession No. HM175885, AF531433, AF091507, AY805221) and Riems strain (U45477). Twelve pairs of primers were designed according to the nucleotide difference between the Shimen and the C-strain using Primer Premier 6.0, and finally two pairs of primers were proved to be available. The primers for the Shimen strain (Accession No. AF092448) were S-F: 5’-GGGCGGCAAAACATCAAATAC-3’ and S-R: 5’-CTCGTCGTGCCGGACAGATT-3’, with an expected product of 438 bp. The primers for the C-strain (Accession No. AF091507) were C-F: 5’-CATGGAAGGGCTGGGCA-3’ and C-R: 5’-CACAAATTTCTTCCGTT-3’, with an expected product of 265 bp.

### Samples

A total of 65 samples were collected, including 5 lymph nodes samples from non-CSF pigs, 5 spleens and whole blood samples from CSFV-infected experimental pigs which manifested clinical and pathological symptoms of CSF, 10 whole-blood samples from HCLV immunized pigs 7–21 d post-immunization, 42 whole-blood samples from clinically healthy pigs from three farms in Shaanxi Province and three cell cultures of PK-15, ST, SUVEC [19], which were simultaneously infected with Shimen and the C-strain.

Care of laboratory animals and animal experimentation were performed in accordance with the animal ethics guidelines and approved protocols. All animal experiments were approved by the Animal Ethics Committee of Northwest A&F University.

### RNA extraction and cDNA synthesis

RNA was extracted from infected cell cultures, blood (100 μL) or tissue (100 mg) using TRIzol® reagent (Life Technologies, Shanghai, China), according to the manufacturer’s instructions. The total RNA was measured with NanoDrop2000 Spectrophotometers (Thermo Fisher, Shanghai, China), according to the instructions. Reverse transcription (RT) was performed using PrimeScript™ 1st Strand CDNA Synthesis Kit (TaKaRa, Dalian, China) with a final volume of 10 μL.

### Duplex PCR for distinguishing shimen and C-strain of CSFV

A duplex PCR for distinguishing the Shimen strain from the C-strain was performed with a total volume of 20 μL, including 2 × PCR Mix 10 μL (Cwbiootech, Beijing, China), cDNA reversed from the co-infected cells 2 μL, each of four primers (10 μmol/L) S-F/S-R and C-F/C-R 0.5 μL and 6 μL of ddH2O. The amplification conditions were as follows: 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min (Bio-Rad S1000, USA). Meanwhile, single PCR was also performed with corresponding cDNA and specific primers. PCR products (10 μL) were visualized by electrophoresis in a 2% (w/v) agarose gel.

The PCR products were purified and sequenced (TaKaRa, Dalian, China) for alignment.

### Specificity of the duplex RT-PCR

The duplex RT-PCR was evaluated for its specificity by testing the Shimen strain, the C-strain and several porcine original viruses including PRV, PCV2, PRRSV, PPV and TGEV. RNA or DNA was extracted (by using TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Ver.5.0) from infected cell cultures.

### Sensitivity test of the duplex RT-PCR

Serial 10-fold dilutions of C-strain or Shimen genomic RNA (10^-1–10^-6) extracted from infected ST cells were subjected to amplification by duplex RT-PCR.

### Repeatability of the duplex RT-PCR

Reproducibility tests of the duplex RT-PCR were operated in triplicate by testing three different titers of cell cultures infected with the Shimen strain and the C-strain.
Applicability of the duplex RT-PCR in the samples

CSFV was detected in the 65 collected samples by using the duplex RT-PCR procedure and the real-time RT-PCR [iCycler iQ5 Real-time Quantitative PCR; Bio-Rad, USA]. The amplification conditions of real-time RT-PCR were as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 56 °C for 1 min and a final extension at 72 °C for 5 min (containing 1 μL of cDNA, 10 μL 2 × Mixture (Cwbiotech China), 0.5 μL of primer solution and 8 μL of water).

Results and discussion

Detection of CSFV by RT-PCR

The RNA of the Shimen strain and the C-strain was detected by using single RT-PCR with designed primers. Fragments of 265 bp or 438 bp were amplified (Figure 1A and Figure 1B). A strong fluorescent signal was also detected in Shimen or C-strain infected cells with real-time RT-PCR (Figure 2).

The sequence alignment of the PCR products showed that the PCR product of the Shimen strain was 98.4% homologous to AF092448, and that of the C-strain was 99.6% homologous to AF091507.

Specificity of duplex RT-PCR

After optimization, the duplex RT-PCR reaction system was determined to be 20 μL, with the primers concentration being 0.5 μmol/L and the annealing temperature, 54 °C. Two bands of 438 and 265 bp were detected simultaneously from the mixed RNA of the Shimen strain and the C-strain (Figure 3). As expected, the PCR bands from a virulent CSFV isolate were similar to that of the

Figure 1. Detection of CSFV C strain (A) or Shimen strain (B) by a single RT-PCR.

Figure 2. Detection of CSFV in Shimen or C-strain infected cells by real-time RT-PCR.
Shimen strain, and the amplification pattern from a commercial attenuated vaccine was similar to that of the C-strain (Figure 4). No PCR bands were obtained from TGEV, PRV, PCV2, PRRSV and PPV-infected cells (Figure 5).

Considering the comparable serology presentation of the swine infected with virulent and attenuated CSFV [20–22], a rapid, sensitive and specific method for distinguishing virulent CSFV from vaccine virus is justified to be of key importance [23]. In this respect, the diagnostic method described here would be significant and useful.

Sensitivity of the duplex RT-PCR

Serial 10-fold dilutions of C-strain, Shimen and C+Shimen genomic RNA ($10^{-1}$ – $10^{-6}$) were subjected to the duplex RT-PCR. The minimum detectable quantity was 0.0616 pg for the C-strain RNA, 0.0844 pg for Shimen RNA (Figure 6A,B), and a similar result was observed for C+Shimen RNA. Three independent repetitions showed consistent results.

Applicability of the duplex RT-PCR

The 65 collected samples were detected by the developed duplex RT-PCR and the results showed that 8 samples were tested to be Shimen-positive from CSFV infected pigs and cell cultures; 55 samples were tested to be HCLV-positive (1 infected pig, 51 non-infected pigs and 3 cell cultures). The obtained results were consistent with those determined by real-time RT-PCR developed in previous studies for CSFV detection (Table 1).

The diagnostic sensitivity estimates for this duplex RT-PCR resulted in 100% sensitivity for detection of virulent CSFV and 96.4% for the vaccine strain. Additionally,
differentiate pigs infected with virulent CSFV from China (spring and autumn), and the vaccine virus is ability of the test.

References

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Table 1. Detection of CSFV in different samples with the duplex RT-PCR and real-time RT-PCR.

| Samples delivered | Types of samples | Number of samples | Detection by RT-PCR | Detection by real-time RT-PCR |
|-------------------|------------------|-------------------|---------------------|-------------------------------|
|                   |                  |                   | Shimen-like  | HCLV-like  | Shimen | HCLV |
| Non-CSF pig       | Lymph nodes      | 5                 | 0          | 3          | 0      | 3    |
| Infected pigs     | Spleen/ blood    | 5                 | 5          | 1          | 5      | 1    |
| Immunized pigs    | Blood            | 10                | 0          | 9          | 0      | 10   |
| Clinical healthy pigs | Blood      | 42                | 0          | 37         | 0      | 38   |
| Co-infected cells | Cells            | 3                 | 3          | 3          | 3      | 3    |
| Total             |                  | 65                | 8          | 53         | 8      | 55   |

Conclusions

The developed duplex RT-PCR was demonstrated to be a useful method for rapid detection and differentiation of pathogenic CSFV and vaccine strains. The protocol presented here is a rapid, sensitive and specific method to differentiate pigs infected with virulent CSFV from immunized pigs.

Disclosure statement

The authors declare that they have no competing interests.

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References

[1] Sawford K, do Karmo A, da Conceicao F, et al. An investigation of classical swine fever virus seroprevalence and risk factors in pigs in Timor-Leste. Prev Vet Med. 2015;122 (1–2):99–106.

[2] Meuwissen E. Richesse oblige: la Belle Epoque des grandes fortunes. 2nd ed. Bruxelles: Racine; 1999.

[3] de Smit AJ. Laboratory diagnosis, epizootiology, and efficacy of marker vaccines in classical swine fever: a review. Vet Quart. 2000;22(4):182–188.

[4] Colijn EO, Bloemraad M, Wensvoort G. An improved ELISA for the detection of serum antibodies directed against classical swine fever virus. Vet Microbiol. 1997;59(1):15–25.

[5] van Oirschot JT. Vaccinology of classical swine fever: from lab to field. Vet Microbiol. 2003;96(4):367–384.

[6] Xia H, Wahlberg N, Qiu HJ, et al. Lack of phylogenetic evidence that the Shimen strain is the parental strain of the lapinized Chinese strain (C-strain) vaccine against classical swine fever. Arch Virol. 2011;156(6):1041–1044.

[7] Tu C, Lu Z, Li H, et al. Phylogenetic comparison of classical swine fever virus in China. Virus Res. 2001;81(1–2):29–37.

[8] Huang YL, Deng MC, Wang FL, et al. Lack of phylogenetic evidence of wild-type and HCLV vaccine strains of classical swine fever virus. Arch Virol. 2014;179:1–11.

[9] von Rosen T, Rangelova D, Nielsen J, et al. DIVA vaccine properties of the live chimeric pestivirus strain CP7 – E2gif. Vet Microbiol. 2014;170(3–4):224–231.

[10] Gong ML, Peng BY, Liu JJ, et al. Cloning and sequencing of full-length cDNA of classical swine fever virus LPC strain. Virus Genes. 2001;23(2):187–192.

[11] Wu HX, Wang JF, Zhang CY, et al. Attenuated lapinized Chinese strain of classical swine fever virus: complete nucleotide sequence and character of 3’-noncoding region. Virus Genes. 2001;23(1):69–76.

[12] Cheng D, Zhao JJ, Li N, et al. Simultaneous detection of classical swine fever virus and North American genotype Porcine reproductive and respiratory syndrome virus using a duplex real-time RT-PCR. J Virol Methods. 2008;151(2):194–199.

[13] Huang YL, Deng MC, Pan CH, et al. Development of a reverse transcription multiplex real-time PCR for the detection and genotyping of classical swine fever virus. J Virol Methods. 2009;160(1–2):111–118.

[14] Leifer I, Everett H, Hoffmann B, et al. Escape of classical swine fever C-strain vaccine virus from detection by C-strain-specific real-time RT-PCR caused by a point mutation in the primer-binding site. J Virol Methods. 2010;166 (1–2):98–100.

[15] Wen GY, Zhang T, Yang J, et al. Development of a triplex TaqMan real-time RT-PCR assay for differential detection of wild-type and HCLV vaccine strains of classical swine fever virus and bovine viral diarrhea virus 1. Res Vet Sci. 2012;92(3):512–518.

[16] Huang YL, Deng MC, Wang FL, et al. Development of a primer-probe energy transfer real-time PCR assay for detection of classical swine fever virus. J Virol Methods. 2010;168(1–2):259–261.
[18] Ning P, Li H, Liang W, et al. Detection and differentiation of classical swine fever virus strains C and Shimen by high-resolution melt analysis. J Virol Methods. 2013;194(1–2):129–131.

[19] Hong HX, Zhang YM, Xu H, et al. Immortalization of swine umbilical vein endothelial cells with human telomerase reverse transcriptase. Mol Cells. 2007;24(3):358–363.

[20] Rossi S, Staubach C, Blome S, et al. Controlling of CSFV in European wild boar using oral vaccination: a review. Front Microbiol. 2015 [cited 2016 Dec 14];6:1141. doi: 10.3389/fmicb.2015.01141.

[21] Schroeder S, von Rosen T, Blome S, et al. Evaluation of classical swine fever virus antibody detection assays with an emphasis on the differentiation of infected from vaccinated animals. Rev Sci Tech Oie. 2012;31(3):997–1010.

[22] Blome S, Gabriel C, Beer M. Possibilities and limitations in veterinary vaccine development using the example of classical swine fever. Berl Munch Tierarztl. 2013;126(11–12):481–490.

[23] Beer M, Goller KV, Staubach C, et al. Genetic variability and distribution of Classical swine fever virus. Anim Health Res Rev. 2015;16(1):33–39.

[24] Pan CH, Jong MH, Huang YL, et al. Rapid detection and differentiation of wild-type and three attenuated lapinized vaccine strains of classical swine fever virus by reverse transcription polymerase chain reaction. J Vet Diagn Invest. 2008;20(4):448–456.

[25] Renson P, Le Dimna M, Keranflech A, et al. CP7-E2alf oral vaccination confers partial protection against early classical swine fever virus challenge and interferes with pathogeny-related cytokine responses. Vet Res. 2013 [Cited 2016 Dec 14];44:9. doi: 10.1186/1297-9716-44-9.