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Rapid detection and differentiation of wild-type and attenuated lapinized vaccine strains of classical swine fever virus by reverse-transcription polymerase chain reaction

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Running title: Rapid RT-PCR differentiation of wild and vaccine-type CSFV

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Summary

A simple one-step reverse transcription-polymerase chain reaction (RT-PCR) method was developed based on T-rich insertions in the viral genome for simultaneous detection and differentiation of wild-type and vaccine strains of classical swine fever virus (CSFV). The CSFV-specific primers were designed to contain the sequences of the T-rich insertion sites that exist uniquely in the 3' nontranslated regions (3' NTR) of the genome of lapinized CSFV vaccine strains. Using a one-step RT-PCR or a semi-nested RT-PCR followed by an agarose gel electrophoresis or a multi-capillary electrophoresis, the wild-type and lapinized vaccine strains of CSFV in clinical samples could be detected and accurately distinguished. These assays can be applied to at least three attenuated lapinized vaccine strains, LPC (lapinized Philippines Coronel), HCLV (hog cholera lapinized virus), and C (Chinese)-strain. The detection limit of the wild-type virus was 6.3 $TCID_{50}$ (50% tissue culture infective dose)/ml for RT-PCR and 0.63 $TCID_{50}$/ml for semi-nested RT-PCR. In previous studies, notable T-rich insertions of 12–13 nucleotides (nts) were found in the 3' NTR of the genome of lapinized vaccine strains of CSFV. However, this study discovered that two T-rich insertions, 42 and 36 nts in length, are present in the viral genome of lapinized vaccine strains LPC/PRK (primary rabbit kidney) and LPC/TS (Tam-Sui), respectively. These T-rich insertions of 12, 36, and 42 nts length increases the size of PCR fragments, which are favorable genetic markers for rapid detection and differentiation of wild-type and different lapinized vaccine strains of CSFV.

Keywords: *Classical swine fever virus; lapinized vaccine strains; nontranslated region; reverse transcription polymerase chain reaction.*

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Introduction

Classical swine fever (CSF), previously referred to as hog cholera, is a highly contagious and significant infectious disease of swine and wild boar caused by CSF virus (CSFV). Classical swine fever virus belongs to the genus *Pestivirus* within the family *Flavivirida* [29]. The other 2 members within the genus *Pestivirus*, namely bovine viral diarrhea virus (BVDV) of cattle and border disease virus (BDV) of sheep, are also important animal pathogens that can infect pigs naturally [24]. Classical swine fever virus isolated around the world has been tentatively divided into 3 major genetic groups, each with 3–4 subgroups: 1.1, 1.2, 1.3; 2.1, 2.2, 2.3; 3.1, 3.2, 3.3, 3.4 [23]. Phylogenetic analysis of field isolates of CSFV showed 4 distinct CSFV genotypes, subgroups 2.1a, 2.1b, 2.2 and 3.4, all of which exist in Taiwan [21].

Attenuated vaccine strains of CSFV can persist in tissue or blood of piglets for a period of time after immunization. In piglets inoculated with the C (Chinese)-strain vaccine virus, the virus can be detected in either tonsils or blood samples at 2–16 days postvaccination (DPV) [16]. The C-strain can be detected in organs at 8 DPV in domestic pigs and 9 DPV in wild boars [8]. The PAV-250 vaccine virus, a strain employed in South America, persisted in tonsils of vaccinated pigs until 28 DPV, as detected both by reverse transcription-polymerase chain reaction (RT-PCR) and by a fluorescent antibody test (FAT) [6]. In weaning pigs vaccinated intramuscularly with C-strain vaccine virus, the virus genome can be consistently detected in tonsils up to 42 DPV by real-time RT-PCR [10].

Molecular analysis of genetic diversity has enabled differentiation of closely related virus strains. Previously, differentiation of CSFV from BVDV and BDV was achieved by RT-PCR [4, 9, 30], RT-PCR followed by restriction endonuclease analysis [27], RT-PCR followed by a hybridization assay [1], or real-time RT-PCR [18, 25]. Genetic grouping of CSFV has also been performed using RT-PCR followed by restriction fragment length polymorphism of the E2 gene of the viral genome [22]. The use of RT-PCR amplification followed by restriction enzyme digestion to differentiate between wild-type and vaccine strains of CSFV has been reported [28, 33]. Recently, RT-PCR amplification followed by direct sequencing of the amplicons has been the most widely used method for phylogenetic analysis of CSFV and for differentiation of wild-type CSFV from vaccine strain [3, 17, 21, 23]. An RT-PCR amplification combined with TaqMan minor-groove-binding (MGB) probes has been reported to distinguish between Korean wild-type and a live attenuated vaccine strain of CSFV (LOM) by single nucleotide difference [5]. A multiplex nested RT-PCR [13] and a multiplex real-time RT-PCR [34] for the detection and differentiation of wild-type viruses from the C-strain vaccine virus have also been described.

The attenuated lapinized vaccine strains of CSFV are currently the most widely used in the world for immunizing pigs against CSFV. Different attenuated lapinized vaccine strains, namely LPC (lapinized Philippines Coronel), HCLV (hog cholera lapinized virus), Riems C (Chinese), and C (Chinese)-strain, are used in different countries. Sequencing of full-length complementary deoxyribonucleic acid (cDNA) of 3 vaccine strains (C-strain, HCLV, and LPC) found that different insertions of 12–13 nucleotides (nts) in length existed in the 3' nontranslated region (3' NTR) of the viral genome [19, 31, 32]. Sequence alignment of the 3' NTRs of CSFV showed that the 12–14-nt T-rich insertions exist in 4 vaccine strains (Porcivac, Rovac, Russian LK, and the original Chinese vaccine) [2]. The unique 12–14-nt T-rich insertions, which are absent from the genome of wild-type CSFV, could be a genetic marker for the vaccine strains, and thus a method based on this difference could be developed to enable rapid differentiation between wild-type and lapinized vaccine strains of CSFV by RT-PCR assay. In the current study, a simple, rapid RT-PCR assay was developed, and the usefulness of this method in routine diagnosis of CSFV infection was determined. This assay was not only able to detect CSFV with high sensitivity, but was also able to distinguish wild-type viruses from lapinized vaccine strains.

Materials and methods

Viruses and vaccine strains

Viruses and vaccine strains used in this study are listed in Table 1.

Clinical samples

A total of 248 clinical samples from different vaccinated farms were submitted to the Animal Health Research Institute (AHRI, Tamsui, Taiwan) for routine CSF diagnosis by local animal disease control centers (LADCCs) between 2004 and 2007. All specimens were prepared as a 10% (w/v) emulsion by homogenizing tonsils and lymph nodes in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO. USA), and were tested by RT-PCR and virus isolation (VI). The results of the VI were confirmed by FAT staining 2 days after inoculation of the specimen emulsions onto cell cultures.

Primer design

Partial NS5B and 3' NTR sequences of 72 CSFV, 11 BDV, 15 BVDV-1, and 8 BVDV-2 strains published in GenBank were aligned using ClustalV of MegAlign 5.03 software (DNASTAR, Inc., Madison, WI, USA). Two sets of degenerate primers were designed based on the conserved NS5B and 3' NTR regions in the 72 CSFV sequences. To distinguish between wild-type and attenuated lapinized vaccine strains of CSFV, the primers were designed to encompass the T-rich insertion site that is unique to the lapinized CSF vaccine strains.

Two forward primers, C5 (5'-GTAGCAAGACTGGRAAYAGGTA-3', Y = C or T, R = A or G; corresponding to Alfort/187 strain 11874 to 11895) and C3 (5'-ACCCTRTTGTARATAACACTA-3'; corresponding to Alfort/187 strain 12106 to 12126), and 1 reverse primer, C6 (5'-AAAGTGCTGTTAAAAATGAGTG-3'; corresponding to Alfort/187 strain 12240 to 12219), were designed. The primer pair C5/C6 was expected to generate long amplification products of 367 and 379 base pairs (bp) from wild-type and lapinized vaccine viruses, respectively. The other primer pair C3/C6 was expected to generate short amplification products of 135 and 147 bp from wild-type and lapinized vaccine viruses, respectively. In an attempt to increase the sensitivity of the test, the primer pair C3/C6 was also used as an internal primer set in combination with C5/C6 for semi-nested RT-PCR amplification.

RT-PCR and semi-nested RT-PCR amplification

Viral RNA was extracted directly from 100 µl of the 10% (w/v) emulsion of tissue specimens, diluted vaccines, sera samples, or cell culture viruses using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription (RT) and subsequent PCR were performed in one tube with a single reaction buffer. The RT-PCR reaction was carried out in a total volume of 50 µl. The reaction mixture contained 1 U of Supertherm DNA Polymerase (JMR Holdings, Kent, UK), 10× buffer supplied by the manufacturer (containing 1.5 mM magnesium chloride), 8 U of RNase inhibitor (Promega, Madison, WI, USA), 2 U of AMV (Avian myeloblastosis virus) reverse transcriptase (Promega, USA), 10 pmole of each primer, 0.1 mM of each dNTP (deoxyribonucleotide triphosphate) (Promega, USA), and 5 µl of RNA sample. The single-step RT-PCR amplification was carried out with the GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA) using the following program: 40 min at 42°C for RT, denaturation for 3 min at 94°C, followed by PCR with 35 cycles of denaturation for 40 sec at 94°C, annealing for 40 sec at 55°C, and extension for 40 sec at 72°C. A final extension step was performed at 72°C for 7 min. When higher sensitivity was required, a semi-nested RT-PCR amplification was performed in a similar manner by using 1 µl of RT-PCR amplicons and semi-nested primers C3/C6 in the second reaction.

Detection of CSFV in sera samples from experimentally infected pigs by RT-PCR and semi-nested RT-PCR

Four 8-week-old specific pathogen-free (SPF) pigs were inoculated intramuscularly with whole blood containing a titer of 10^5 $TCID_{50}$ (50% tissue culture infective doses) of wild-type CSFV (92-TC1 strain). Serum samples were collected at 0, 1, 2, 3, 5, 7, 10, and 12 days postinoculation, and the presence of CSFV in these samples was determined by RT-PCR and semi-nested RT-PCR amplification.

Analysis of amplified products by agarose gel electrophoresis

The amplified products were analyzed by electrophoresis through 4% (for primer pair C3/C6) or 2% (for primer pair C5/C6) agarose gels (SFR™ Biotechnology Grade; AMRESCO, Solon, OH, USA) containing 1x SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) in 1× Tris-acetate-EDTA (ethylenediamine tetra-acetic acid; TAE) solution.

Analysis of amplified products by multi-capillary electrophoresis

The RT-PCR amplification products were analyzed on an eGene HDA-GT12 system with a multi-capillary gel cartridge GCK-5000F (eGene Inc., Irvine, CA, USA) [15]. Ten microliters of the amplicons were mixed with 15 µl Tris-EDTA (TE) buffer in the instrument sample tray. The mixture was automatically injected into the capillary channel and subjected to electrophoresis according to the eGene operation manual. Separations were performed by AM420 method using a 10-sec injection time and a 420-sec separation time. The molecular weight and the concentration of the amplicons were determined with reference to a pGEM DNA size marker (eGene Inc., Irvine, CA, USA).

Sensitivity and specificity assays

To examine the sensitivity of RT-PCR and semi-nested RT-PCR reactions, a cell culture supernatant containing CSFV field isolate, 94-O1030, with an FAT titer of $10^{3.8}$ $TCID_{50}$/ml was used for sensitivity test based on ten-fold dilutions. The total RNA was extracted from each dilution and used in RT-PCR and semi-nested RT-PCR assays. In the test for the specificity of the assay, 2 BVDV-1 strains, 2 BVDV-2 strains, BDV, PRRSV, PCV2, and SIV were used.

Nucleotide sequencing of the 3' NTR of vaccine strains and wild-type CSFV

The RT-PCR products amplified by primer pair C5/C6 were purified using the QIAQuick Purification Kit (Qiagen Inc., Valencia, CA, USA) without gel extraction. DNA sequencing of the RT-PCR products were determined by direct sequencing using the BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems, Foster City, CA, USA) with the original RT-PCR primers. However, direct sequencing on a long T-rich fragment was always failed due to the slippage effect [7]. To overcome this difficulty, 2 RT-PCR products amplified from long T-rich insertions of the LPC/PRK and LPC/TS vaccine strains were cloned using a pBAD/Thio TOPO (topoisomerase I) TA (*Taq*-amplified) Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Ten

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different plasmid clones for each LPC/PRK and LPC/TS strain were chosen for DNA sequencing using a standard vector primer.

Results

Specificity of the RT-PCR and semi-nested RT-PCR

Using the primer pair C5/C6, 158 wild-type CSFV, ALD, and CAP strains, and 5 lapinized vaccine strains (LPC/AHRI, LPC/TS, LPC/PRK, HCLV, and C-strain), were successfully amplified by RT-PCR. As expected, the wild-type CSFV had PCR products of 367 base pairs (bp) whereas 3 vaccine viruses (LPC/AHRI, HCLV, and C-strain) had PCR products of 379 bp. The slightly larger products of LPC/PRK and LPC/TS strains could be distinguished from those of the wild-type viruses in 2% agarose gels, but the products of 3 vaccine viruses (LPC/AHRI, HCLV, and C-strain) could not be distinguished from the wild-type viruses (Fig. 1A). Using the primer pair C3/C6, all the aforementioned wild-type and lapinized vaccine strains were successfully amplified. As expected, 135 bp fragments were generated from the wild-type CSFV. However, different amplification fragments ranging from 147–177 bp were generated from five vaccine viruses. The products of vaccine viruses could be easily distinguished from those of the wild-type CSFV in 4% agarose gels (Fig. 1B). In an attempt to increase the sensitivity, a semi-nested RT-PCR assay was also developed. The primer pair C3/C6 was used as an internal primer set for the semi-nested RT-PCR. In the semi-nested RT-PCR, 158 wild-type CSFV, ALD, and CAP strains, and 5 lapinized vaccine strains, generated PCR products of the expected sizes as in the RT-PCR (Fig. 1C). Other pestiviruses including BVDV/NADL, BVDV/Nose, BVDV-1 vaccine strain, BVDV-2 vaccine strain, and BDV, and other common swine viruses, such as PRRSV, PCV-2, and SIV, were not amplified by these 2 sets of primers (data not shown).

Detection and differentiation of wild-type and attenuated lapinized vaccine strains from clinical samples by RT-PCR

A total of 248 clinical samples were tested in this study. Thirty-five of these samples were positive for CSFV by RT-PCR. Of these 35 samples, 9 were identified persistence of wild-type CSFV (Table 2) and 26 were identified persistence of lapinized vaccine strains (Fig. 2). In VI, only 19 of the 248 clinical samples were positive for CSFV by FAT staining. Of these 19 samples, 9 were wild-type CSFV, and 10 were vaccine viruses. These results showed that the RT-PCR assay was more sensitive than VI.

Multi-capillary electrophoresis analysis

Using a multi-capillary electrophoresis system, the long RT-PCR products amplified by the primer pair C5/C6 could be easily separated into the wild-type and the vaccine viruses even when the difference is as small as 12 nts. Figure 3 showed the separation of 4 lapinized vaccine strains (LPC/AHRI, LPC/PRK, LPC/TS, and HCLV) and the wild-type CSFV represented by 5 different viral genotypes in a slab gel view.

Sensitivity of the RT-PCR and semi-nested RT-PCR

In RT-PCR using primer pairs C5/C6 and C3/C6, the detection limits for wild-type CSFV were 6.3 $TCID_{50}$/ml and 63 $TCID_{50}$/ml, respectively. This shows that RT-PCR with primer pair C5/C6 was 10 times more sensitive than with primer pair C3/C6. In semi-nested RT-PCR using the primer pair C3/C6, the detection limit of wild-type CSFV was 0.63 $TCID_{50}$/ml. Based on these results, semi-nested RT-PCR was 10–100 times more sensitive than RT-PCR.

Nucleotide sequence comparison of the 3' NTR of wild-type CSFV and vaccine viruses

RT-PCR products of 9 wild-type and 8 vaccine-type CSFV obtained from the 248 clinical samples were sequenced using the C5 and C6 primers. The results of DNA sequencing showed that the 9 wild-type CSFV samples belong to CSFV subgroup 2.1a, and the 8 vaccine viruses were of the LPC strain belonging to subgroup 1.1 (data not shown). In addition, 3 vaccine strains of subgroup 1.1 (LPC/AHRI, LPC/PRK, and LPC/TS) and wild-type CSFV of 4 different genotypes (92-TC1 of subgroup 2.1a, 90-YL1 of subgroup 2.1b, 84-KS1 of subgroup 2.2, and 85-12A of subgroup 3.4) were also sequenced. Sequences of these 7 viral strains were submitted to GenBank: 85-12A (EU107748), 84-KS1 (EU107749), 92-TC1 (EU107750), 90-YL1 (EU107751), LPC/AHRI (EU107752), LPC/TS (EU107753), and LPC/PRK (EU107754). The results of direct sequencing of the RT-PCR products showed that the wild-type CSFV lacked T-rich insertions and the vaccine strain LPC/AHRI contained a 12-nt T-rich insertion. The results of sequencing 10 different plasmid clones showed that the LPC/PRK and LPC/TS contained a T-rich insertion of size in the range of 32–42 and 27–36 nts, respectively. The two clones containing the longest T-rich insertions of 42 and 36 nts were derived from the LPC/PRK and LPC/TS strains, respectively. They were sequenced using both forward and reverse primers, and identical results were obtained with both primers. A sequence alignment of the T-rich insertions of 3 vaccine strains, LPC/AHRI, LPC/PRK, and LPC/TS is shown in Figure 4. The length of T-rich insertions of these 3 vaccine strains are 12, 36, and 42 nts, respectively.

Discussion

Classical swine fever is a highly contagious and often fatal disease of swine. The attenuated lapinized CSF vaccine strains such as C-strain, LPC, and HCLV can induce virtually complete protection against the disease [14, 19, 26, 32]. Since the attenuated vaccine viruses can persist in vaccinated pigs for a long period of time [10, 16], they may interfere with the use of laboratory diagnostic tools to detect wild-type CSFV. In Taiwan, the ability to rapidly distinguish between wild-type and vaccine strains of CSFV has been of essential importance for laboratory diagnosis of CSF, because extensive vaccination against CSF has been enforced. RT-PCR followed by DNA sequencing or digestion with restriction enzymes has once been the main method used in Taiwan to detect CSFV and to exclude the interference of vaccine viruses [20, 21]. To simplify the diagnosis of CSF, a simple one-step, single-tube RT-PCR assay based on the T-rich insertions for the simultaneous detection and differentiation of wild-type viruses from vaccine strains of CSFV was developed in the current study. RT-PCR products can be analyzed by conventional agarose gel electrophoresis (Figs. 1B, 1C, 2) or automated multi-capillary electrophoresis, the latter of which provides higher resolution and shorter analysis time for rapid differentiation between wild-type and vaccine strains of CSFV (Fig. 3). Results of clinical testing showed that 26 of 248 (10.5%) clinical samples were identified as vaccine viruses by RT-PCR. Similar results were obtained in a previous study in which 18 of 133 (13%) field samples obtained from vaccinated pigs showed the presence of the C-strain vaccine virus by a multiplex nested RT-PCR [13]. Therefore, in countries where live CSF vaccines are used, the possibility of detecting vaccine viruses in clinical samples should be considered in routine diagnosis of CSFV.

In an effort to amplify different genotypes of CSFV without amplifying 2 closely related pestiviruses (BVDV and BDV), 2 sets of degenerate primers were designed based on the conserved regions of the CSFV genome. Five different genotypes of CSFV, comprising 158 wild-type CSFVs (subgroups 2.1a, 2.1b, 2.2, and 3.4), ALD and CAP strains (subgroup 1.1), and 5 lapinized vaccine strains (subgroup 1.1) could be amplified, and wild-type and vaccine viruses could be distinguished using these 2 sets of primers. In contrary, no amplification was observed from other pestiviruses such as BVDV-1, BVDV-2, and BDV, or from other common swine viruses such as PRRSV, PCV-2, and SIV. This demonstrated that the RT-PCR primers used in the current study were highly specific for CSFV. In sensitivity tests, the RT-PCR primer pairs C5/C6 and C3/C6 were each able to detect wild-type CSFV individually at a virus titer of 6.3 and 63 $TCID_{50}$/ml, respectively. Moreover, C3/C6 could be used as a set of semi-nested RT-PCR primers to obtain higher sensitivity. The semi-nested RT-PCR increased the detection limit to 0.63 $TCID_{50}$/ml. Pigs experimentally infected with wild-type CSFV were positive for CSFV as early as 3 DPV by RT-PCR and 2 DPV by semi-nested RT-PCR. The results demonstrated that the RT-PCR assays were highly sensitive and could detect the early stages of CSFV infection.

A one-step RT-PCR assay using TaqMan MGB probes based on single nucleotide difference was developed to distinguish between vaccine- and wild-type CSFV in Korea [5]. The attenuated Korean LOM vaccine strains have a T at nt 220, whereas Korean wild-type viruses and most of the CSFV have a G in the same position. Therefore, it is possible to distinguish them by means of the MGB probes [5]. However, lapinized CSF vaccine strains such as LPC, HCLV, C-strain, and Riem C all contain a G at nt 220. Therefore, the real-time assay described previously [5] does not seem applicable to distinguish between wild-type viruses and lapinized CSF vaccine strains. Another study described a multiplex nested RT-PCR for the detection and differentiation of wild-type viruses from the C-strain based on 2 nucleotide differences (GT vs. AC) in the 3' end of a specific primer [13]. However, the LPC strain and the wild-type CSFV have the same AC allele. Therefore, these primers described previously [13] does not seem applicable to distinguish between wild-type viruses and LPC vaccine strains. A multiplex real-time RT-PCR for quantitative and differential detection of wild-type viruses from C-strain vaccine viruses was also described recently [34]. However, several Taiwanese field strains (P97 and 0406/TWN) show some polymorphisms within the probe sequence. Hence, these Taiwanese field strains probably cannot be detected by the assay [34]. There are 7 vaccine strains containing the 12–14-nt T-rich insertions in the 3' NTR that have been reported [2, 19, 31, 32], and none of the wild-type CSFV strains were ever reported to contain the insertions. Results showed that the RT-PCR assay developed in this study could be applied to at least 3 attenuated lapinized vaccine strains, namely LPC, HCLV and C-strain. The Riems C vaccine strain is a cell culture–adapted derivative of the HCLV strain [32]. Three Riems C sequences deposited in GenBank showed differences in the T-rich region. One of the Reim vaccine strains (U45477) lacks of the T-rich insertion, another Reim vaccine strain (AY259122) contains a T-rich insertion and a deletion, and the third Reim vaccine strain (U45456) contains a 28-nt T-rich insertion (Fig. 4). Therefore, whether the assay can be applied to Riems C vaccine strains is still unknown.

The current study found that the T-rich insertions of the LPC/PRK and LPC/TS strains are longer than those of the strains previously reported. To determine the exact length of the T-rich insertions in the LPC/PRK and LPC/TS strains, plasmid clones prepared from the RT-PCR products from these vaccine strains were sequenced. The results showed that the T-rich insertions of the LPC/PRK and LPC/TS strains were 32–42 nts and 27–36 nts long, respectively. Combining results of DNA

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sequencing and multi-capillary electrophoresis, it was determined that the longest sequences, 42 nts for the LPC/PRK strain and 36 nts for the LPC/TS strain, were the lengths of the T-rich insertions in their genomes.

Two different lengths of T-rich insertions, 42 and 36 nts, were discovered in the descendant of the LPC/China vaccine virus (Fig. 4) in the current study. Attenuation of the lapinized CSFV strain was originally done in Taiwan in the 1950s. A Rovac strain of the lapinized CSFV that had already undergone about 250 serial passages in rabbits in the Lederle Laboratory was given to Dr. Lee by Dr. Coronel AB and introduced into Taiwan from the Philippines by Lee in 1952 [11, 12]. Pigs inoculated with this virus showed severe postvaccination reactions, and some even died of apparent CSF. In order to obtain a safer vaccine strain, the virus was then further serial-passaged through rabbits. After more than 800 passages, the rabbit-adapted virus had completely lost its virulence and become a safe and effective vaccine virus against CSF for pigs. This vaccine virus was designated as the LPC strain or LPC/China strain, and the seed virus was kept at AHRI for the production of live vaccine [14]. The LPC/TS vaccine strain was derived from the LPC/China vaccine strain by adaptation to the PK-15 (porcine kidney) cell line for 10 passages followed by cloning twice at a limiting dilution and subsequent growth for 21 passages in the cell line. Why does the original LPC/China strain contain a 12-nt T-rich insertion while its descendant, LPC/TS, contains a 36-nt T-rich insertion? Tracing back the procedure of vaccine development, it was found that the addition of T-rich insertions occurred during the cloning by limiting dilution rather than in the serial passages in tissue culture. The LPC/PRK vaccine strain also obtained a 42-nt T-rich insertion during cloning by limiting dilution (Frankie Huang, pers. comm. 2007). Based on the fact that the LPC/China vaccine virus has undergone 1,050 serial passages in rabbits, the vaccine virus is not considered as a uniform population. In fact, it contains several different particles that carried T-rich insertions of different lengths. Two tissue culture–adapted lapinized vaccine viruses (LPC/TS and LPC/PRK) carrying 36- and 42-nt T-rich insertions were unexpectedly selected for during vaccine preparation. The LPC/TS strain can be propagated up to $10^{7.3}$ $TCID_{50}$/ml in the PK-15 cell line, and the viral titer is higher than the LPC/China strain grown in PK-15 cells (data not shown). It seems that the function of long T-rich insertions could be to increase the virus titer and result in easier selection in the procedure of cloning by limiting dilution.

In conclusion, a simple one-step RT-PCR assay presented in the current study could provide a rapid and sensitive diagnostic tool for the specific detection of CSFV, and for distinguishing animals infected with wild-type viruses from those vaccinated with lapinized vaccine strains in the field.

Table 1. Viruses and vaccine strains used in this study*

Viruses and vaccine strains tested	Material	Number of units
wild-type CSFV		
Field isolates†	Swine tonsils & lymph nodes	158
Strain ALD‡	Swine blood	1
Strain CAP ¦	IBRS-2 cells	1
Lapinized CSFV vaccine strains§		
LPC/AHRI #	Rabbit spleen & lymph nodes	1
LPC/TS #	PK-15 cells	1
LPC/PRK¶	Primary rabbit kidney cells	1
HCLV **	Cell cultures	1
C-strain $	Cell cultures	1
Other Pestiviruses		
BDV strain 31▲	Primary sheep leptomeningeal cells	1
BVDV-1		
NADL strain	Primary bovine testicle cell	1
Nose strain	Primary bovine testicle cell	1
BVDV-2		
MD strain	Primary bovine testicle cell	1
Vaccine strain ★	Cell cultures	1
Common viruses		
Porcine reproductive and respiratory syndrome virus (PRRSV)	MARC-145 cell	1
Porcine circovirus type 2 (PCV-2)	PK-15 cell	1
Swine influenza virus (SIV) subtype H1N2	MDCK cell	1

* CSFV = Classical swine fever virus; LPC = lapinized Philippines Coronel; AHRI = Animal Heslth Research Institute; TS = Tam-Sui; PRK = primary rabbit kidney; HCLV = hog cholera lapinized virus; C = Chinese; BDV = Border disease virus; BVDV = Bovine viral diarrhea virus; NADL = National Animal Disease Laboratory; MD = mucosal disease.

† These isolates were collected in Taiwan from 1989 to 2003 and were genotyped as subgroups 2.1a, 2.1b, 2.2, or 3.4.

‡ A laboratory reference strain belonging to CSFV subgroup 1.1.

¦ A laboratory reference strain belonging to CSFV subgroup 1.1. This virus strain was isolated from persistently infected IBRS-2 cells.

§Three LPC vaccine strains (LPC/AHRI, LPC/TS, LPC/PRK) were derived from the LPC/China strain.

LPC/AHRI; LPC/TS (Animal Health Research Institute, Tamsui, Taiwan).

¶ LPC/PRK (Formosa Biomedical Technology Corp., Taipei, Taiwan).

** HCLV (China Animal Husbandry industry Co. Ltd., Nanjing, China).

$ C-strain (Pestiffa®, Merial SAS, Lyon, France).

▲ BDV strain 31 (VR-996™ Global Bioresource Center, Manassas, VA, USA)

★Vaccine strain (Bovi-Shield® Gold 4, Pfizer Animal Health, Exton, PA, USA)

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Table 2. Detection of wild-type CSFV in clinical samples collected from nine pig farms during 2004–2007 in Taiwan.

Field isolates	Prefecture	Year of isolation	Subgroup
93-N210	Chanhua	2004	2.1a
93-N956	Taidon	2004	2.1a
93-N1211	Miaoli	2004	2.1a
93-N1251	Sinchu	2004	2.1a
93-N1474	Miaoli	2004	2.1a
94-O446	Sinchu	2005	2.1a
94-O1030	Chanhua	2005	2.1a
95-M06-29	Yunlin	2006	2.1a
ATIT07-6		2007	2.1a

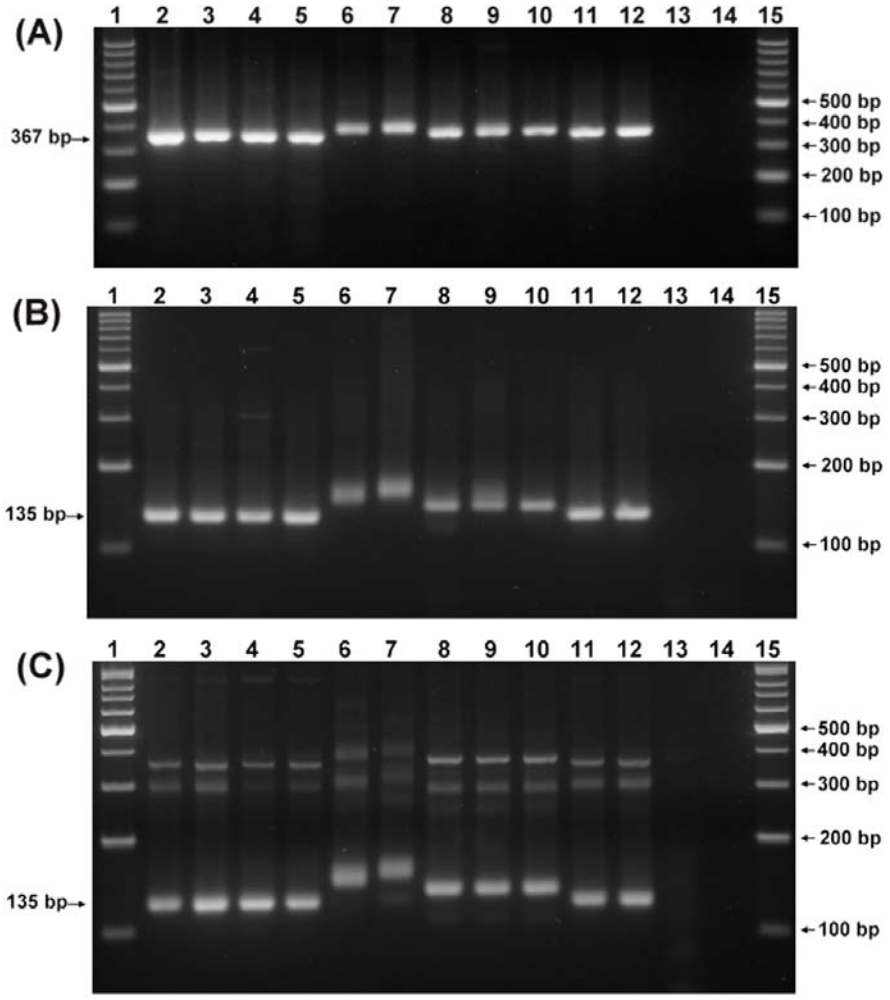

Figure 1. Amplification of different genotypes of classical swine fever virus (CSFV) and 4 lapinized vaccine strains. The reaction products were analyzed by agarose gel electrophoresis. **A,** RT-PCR amplification with the primer pair C5/C6. **B,** RT-PCR amplification with the primer pair C3/C6. **C,** Semi-nested RT-PCR amplification with the primer pair C3/C6. Lanes 1 and 15: 100-bp DNA ladder marker; lane 2: 85-12A; lane 3: 84-KS1; lane 4: 92-TC1; lane 5: 90-YL1; lane 6: LPC/TS; lane 7: LPC/PRK; lane 8: LPC/AHRI; lane 9: HCLV; lane 10: vaccine-type viruses in clinical samples; lane 11: wild-type virus in clinical samples; lane 12: ALD; lane 13: BVDV (bovine viral diarrhea virus)/NADL (National Animal Disease Laboratory); lane 14: negative control. The amplified 367- and 135-bp fragments detected in the wild-type CSFV are indicated by arrows on the left of the gel.

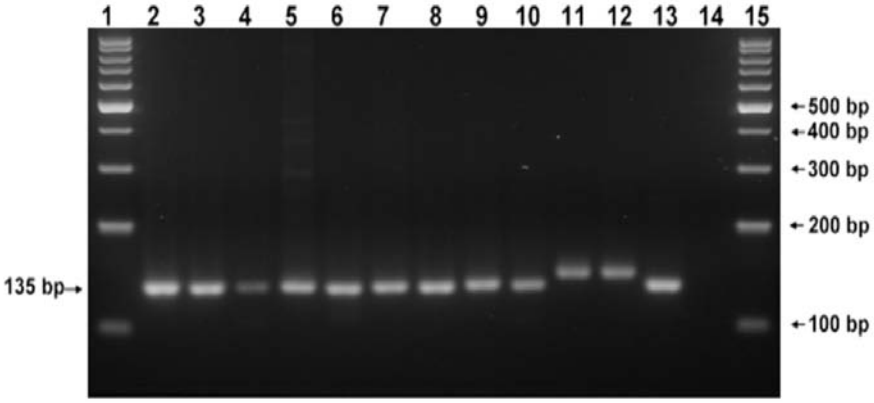

Figure 2. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of clinical samples with the primer pair C3/C6. Lanes 1 and 15: 100-bp DNA ladder marker; lanes 2–10: 9 wild-type viruses in clinical samples collected from 9 pig farms during 2004–2007; lane 11-12: vaccine-type virus in clinical samples; lane 13: ALD; lane 14: negative control. The detected amplified 135-bp fragment from the wild-type classical swine fever virus (CSFV) is indicated by an arrow on the left of the gel.

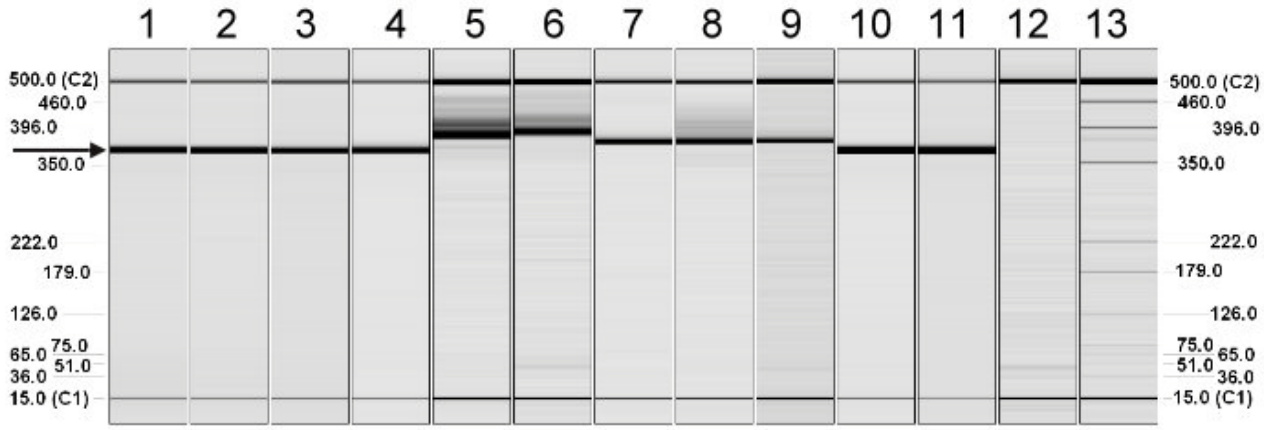

Figure 3. Multi-capillary electrophoresis analysis of T-rich insertions in wild-type and lapinized vaccine strains of classical swine fever virus (CSFV). Separations are aligned by means of bracketing standards C1 (15 bp) and C2 (500 bp) for highly accurate size determination. Lane 1: 85-12A (367 bp); lane 2: 84-KS1 (367 bp); lane 3: 92-TC1 (367 bp); lane 4: 90-YL1 (367 bp); lane 5: LPC (lapinized Philippines Coronel)/TS (Tam-Sui; 403 bp); lane 6: LPC/PRK (primary rabbit kidney; 409 bp); lane 7: LPC/AHRI (Animal Health Research Institute; 379 bp); lane 8: HCLV (hog cholera lapinized virus; 379 bp); lane 9: vaccine-type virus in clinical samples (379 bp); lane 10: wild-type virus in clinical samples (367 bp); lane 11: ALD virulent strain (367 bp); lane 12: negative control; lane 13: pGEM DNA marker. The detected amplified 367-bp fragment from the wild-type CSFV is indicated by an arrow on the left of the gel.

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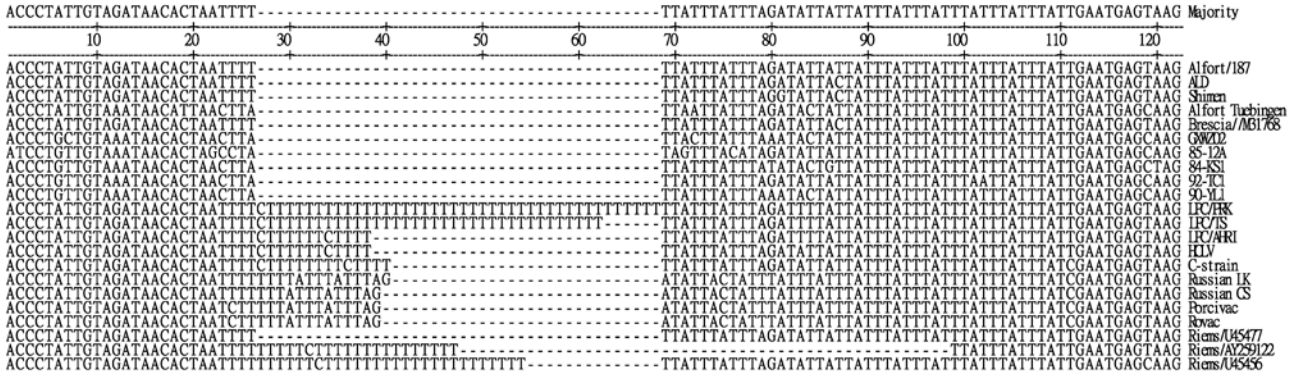

Figure 4. Nucleotide sequence alignment of the T-rich insertion sequences from the 3' NTR of classical swine fever virus (CSFV; bases 12106-12185 from the Alfort/187 sequence). The accession numbers of 22 sequences included in this alignment are as follows: Alfort/187 (X87939), ALD (D49532), Shimen (AF092448), Alfort/Tubingen (J04358), Brescia (M31768), GXWZ02 (AY367767), 85-12A (EU107748), 84-KS1 (EU107749), 92-TC1 (EU107750), 90-YL1 (EU107751), LPC/PRK vaccine strain (EU107754), LPC/TS vaccine strain (EU107753), LPC/AHRI vaccine strain (EU107752), HCLV vaccine strain (AF531433), C-strain vaccine strain (Z46258), Russian LK vaccine strain (AF026718), Russian CS vaccine strain (AF099102), Porcivac vaccine strain (AF026714), Rovac vaccine strain (AF026717), Riems vaccine strain (U45477), Riem vaccine strain (AY259122), and Riems vaccine strain (U45456).

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建立快速區別豬瘟野外毒及兔化豬瘟疫苗毒之 RT-PCR 檢測方法

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摘要　本試驗基於兔化豬瘟疫苗毒核酸序列中有一段 T-rich 插入之特性發展單一步驟 RT-PCR 檢測方法，使用單步驟 RT-PCR 或巢式 RT-PCR 增幅，經傳統洋菜膠或毛細管電泳後，可同時檢測及區別臨床檢體中的豬瘟野外毒及兔化豬瘟疫苗毒。本方法至少可應用於 LPC、HCLV 及 C-strain 等三種兔化豬瘟疫苗毒，對於豬瘟野外毒檢測的敏感度 RT-PCR 及巢式 RT-PCR 分別為 6.3 及 0.63 $TCID_{50}$/ml。前人報告指出，兔化豬瘟疫苗毒核酸序列 3' 端未轉譯區有一段 12 至 13 個 T-rich 插入之特性，然而我們發現 LPC/PRK 及 LPC/TS 兩株疫苗毒 T-rich 插入片段長度分別多達 42 及 36 個核苷酸，這些不同大小的 T-rich 插入片段增加 RT-PCR 產物的大小，可當做很好的基因標記藉以快速區別豬瘟野外毒及不同兔化豬瘟疫苗毒株。

關鍵字：豬瘟野外毒、兔化豬瘟疫苗毒、非轉譯區、反轉錄聚合酶鏈反應。

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