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Generation of a recombinant classical swine fever virus stably expressing the firefly luciferase gene for quantitative antiviral assay

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ABSTRACT

Classical swine fever (CSF), caused by classical swine fever virus (CSFV), is a highly contagious swine disease leading to significant economic losses worldwide. Vaccines are widely used to control the disease, and no CSFV-specific antivirals are currently available. To facilitate anti-CSFV molecule discovery, we developed a reporter virus CSFV-N^{pro}Fluc stably expressing the firefly luciferase (Fluc) gene in the N^{pro} gene. The reporter virus enabled more sensitive and convenient detection of the N^{pro} protein expression and the viral replication by luciferase reporter assay than by traditional methods. The CSFV N^{pro} protein was detectable as early as 4.5 h post-infection. As a proof-of-concept for its utility in rapid antiviral screening, this reporter virus was used to quantify anti-CSFV neutralizing antibodies of 50 swine sera and to assess 12 small interfering RNAs targeting different regions of the CSFV genome. The results were comparable to those obtained by traditional methods. Taken together, the reporter virus CSFV-N^{pro}Fluc represents a useful tool for rapid and quantitative screening and evaluation of antivirals against CSFV.

1. Introduction

Classical swine fever (CSF), which is caused by classical swine fever virus (CSFV), is a highly contagious disease of pigs and a notifiable disease to the World Organization for Animal Health (OIE). The disease causes significant economic losses, representing a constant and serious threat to the pig industry worldwide, particularly in Asia, Latin America, and Eastern Europe (Edwards et al., 2000; Vandeputte and Chappuis, 1999). CSFV is a member of the *Pestivirus* genus within the *Flaviviridae* family and possess a single-stranded, positive-sense RNA genome of approximately 12.3 kb (Pletnev et al., 2011). The genome contains a 5′-untranslated region (5′-UTR), a single large open reading frame (ORF), and a 3′-UTR. The ORF encodes a precursor polyprotein of 3898 amino acids (aa) that is co- and post-translationally processed by viral as well as cellular proteases, giving rise to four structural proteins (C, E^{rns}, E1 and E2) and seven nonstructural proteins (N^{pro}, p7, NS2-3, NS4A, NS4B, NS5A and NS5B) (Moennig, 2000).

Reverse genetics system of positive-sense RNA viruses, which enables generation of infectious viruses from a full-length cDNA clone of the viral genome, is a powerful tool to study molecular details of various aspects of the viral life cycle. A marker virus with the introduction of a tag into the viral genome can be used in the study of viral replication, protein functions and drug discovery (Beer et al., 2007). For example, the marker virus expressing enhanced green fluorescent protein (EGFP) allows rapid identification of viral infection and direct detection of anti-CSFV neutralizing antibodies (NAbs) without immunostaining (Li et al., 2013b). However, an EGFP-based assay requires extensive and costly automated imaging equipment (Li et al., 2013b) and does not fit for high-throughput screening (HTS) assays.

Luminescent reporters provide a viable alternative to fluorescent reporters in HTS assays for chemical biology and drug discovery (Miraglia et al., 2011). They facilitate the development of highly sensitive, cell-based reporter assays (Thorne et al., 2010), eliminate the problem of compound fluorescence (Simeonov et al., 2008) and possess several advantages such as high reliability, convenience and adaptability to HTS assays.

In this study, we generated a reporter CSFV (CSFV-N^{pro}Fluc) stably expressing the firefly luciferase (Fluc) gene as an alternative to the EGFP-tagged CSFV (Li et al., 2013b). We showed that CSFV-N^{pro}-Fluc allows rapid and sensitive detection of the viral protein. Furthermore, CSFV-N^{pro}Fluc was successfully used to quantify NAbs in a 96-well plate format, and assess the effects of small interfering RNAs (siRNAs) targeting different regions of the CSFV genome on the viral replication in a 48-well plate format.

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2. Materials and methods

2.1. Cells and viruses

SK6 and PK-15 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine (Sigma, St. Louis, MO, USA) and incubated at 37 °C with 5% CO_2. The CSFV Shimen strain (GenBank accession number AF092448.2) was the parent virus for generating the reporter virus below. All viruses were propagated in SK6 cells in DMEM supplemented with 4% FBS.

2.2. Generation of a full-length CSFV cDNA clone containing the Fluc gene

The infectious cDNA clone pBRCISM (Li et al., 2013a) (Fig. 1A) containing a full-length cDNA copy of the CSFV Shimen strain was used as the backbone to generate the Fluc reporter virus. The Fluc gene was amplified by PCR from the pGEM-luc vector (Promega, Madison, WI, USA) and introduced into the plasmid pBRCISM. Briefly, the Fluc gene was introduced between aa 13 and 14 of N^{pro} by standard overlapping PCR using the following primer pairs N^{pro}Fluc-1-F (5′-CCC TCG AGA TGC TAT GTG GAC GAG GGC ATG-3′)/N^{pro}Fluc-1-R (5′-CTT TAT GTT TTT GGC GTC TTC CAT GTT TGT TTT GTA TAA AAG TTC AAA-3′), N^{pro}Fluc-2-F (5′-TTG AAC TTT TAT ACA AAA CAA ACA TGG AAG ACG CCA AAA ACA TAA AG-3′)/N^{pro}Fluc-2-R (5′-CCA CTC CCA TTG GTT TTT GTT TCA ATT TGG ACT TTC CGC CCT TC-3′) and N^{pro}Fluc-3-F (5′-GAA GGG CGG AAA GTC CAA ATT GAA ACA AAA ACC AAT GGG AGT GGA G-3′)/N^{pro}Fluc-3-R (5′-CTC TAG AGG GGC CCT ATG GTA GAC CG-3′), and cloned into pBRCISM by homologous recombination, resulting in pBRCISM-N^{pro}Fluc (This construct is available for other scientists upon request) (Fig. 1B). The final construct was verified by sequencing.

2.3. Rescue of recombinant virus

Virus rescue was performed as described previously (Li et al., 2013a). Briefly, SK6 cells grown to 80% confluence were transfected with 2 μg of pBRCISM-N^{pro}Fluc using the X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). After incubation for 6 h at 37 °C in a humidified 5% CO_2 incubator, the transfected cells were washed three times with DMEM and maintained in DMEM supplemented with 4% FBS for 2 d. The recombinant virus was rescued from the transfected cells after four blind passages and analyzed using a commercial CSFV antigen-capture ELISA (IDEXX, Liebefeld-Bern, Switzerland) and verified by sequencing.

2.4. Indirect immunofluorescent assay (IFA)

SK6 cells infected with CSFV were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. The fixed cells were incubated with anti-E2 sera (1:400 dilution in PBS) for 2 h at 37 °C, washed three times with PBS, and incubated with FITC-labeled goat anti-pig IgG (1:100 dilution in PBS) (Sigma, St. Louis, MO, USA) for 1 h at 37 °C. After washing three times with PBS, the cells were examined under the fluorescence microscope (Nikon, TE2000U, Melville, NY, USA) with a video documentation system.

2.5. Virus titration

The titers of CSFV-N^{pro}Fluc and Shimen strain were determined by IFA as described previously (Li et al., 2013a). Briefly, the 10-fold diluted viruses were used to infect SK6 or PK-15 cells in 96-well plates. The viral titers were determined at 48 h post-infection (hpi) by IFA and expressed as median tissue culture infective dose ($TCID_{50}$)/ml, according to the method of Reed and Münch (1938).

2.6. Western blot analysis

SK6 cells were lysed with NP-40 buffer (50 mM Tris (pH 8), 150 mM NaCl, 0.5% NP-40, and 0.5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml protease inhibitor cocktail (Roche, Mannheim, Germany) for 1 h at 4 °C. An equal volume of each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes (Pall, Dreieich, Germany). The membranes were blocked with 5% skim milk in PBS containing 0.5% Tween (PBST) for 1 h at room temperature and incubated with home-made anti-N^{pro} mouse sera (1:400 dilution in PBS). β-Tubulin,

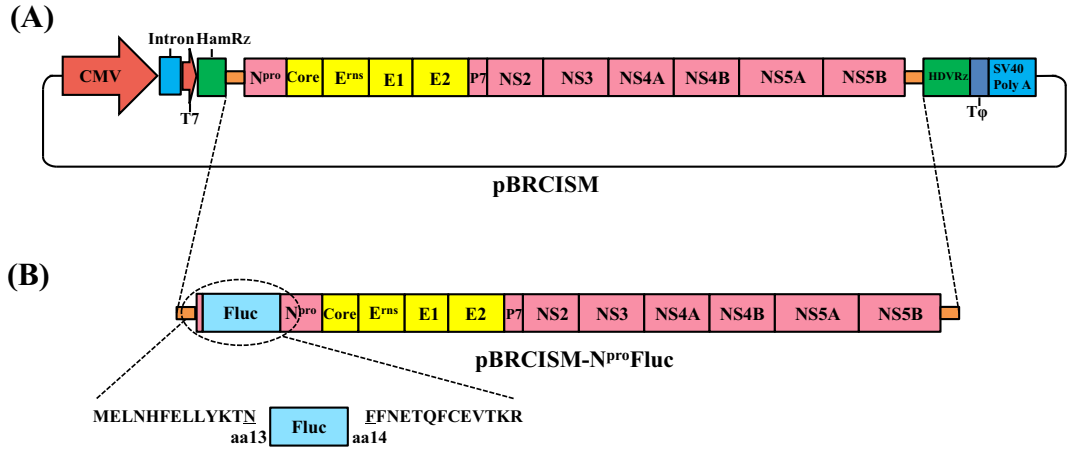

Fig. 1. Schematic representation of the cDNA clones pBRCISM (A) and pBRCISM-N^{pro}Fluc (B). The infectious cDNA clone of the CSFV Shimen strain was used as the backbone for construction of the cDNA clone of CSFV harboring the Fluc gene, which was introduced into the N^{pro} gene by overlapping PCR as described in Materials and methods. Coding regions are depicted as broad bars together with their respective gene names (structural proteins are showed in yellow and nonstructural proteins in red). Untranslated regions (UTR) are depicted as narrow bars using the orange color. The inserted Fluc gene is shown in light blue with the blowups showing the amino acids surrounding the inserted site. CMV, cytomegalovirus immediate early promoter; Intron, chimeric intron; HamRz, hammerhead ribozyme; HDVRz, hepatitis delta virus ribozyme; Tφ, T7 terminator sequence; polyA, SV40 late polyadenylation signal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

used as an internal control, was detected with a mouse anti-β-tubulin monoclonal antibody (1:1000) (Sigma, St. Louis, MO, USA). The blots were incubated with IRDye 800CW goat anti-mouse IgG (H + L) (1:10,000) (926-32210; LiCor Biosciences, Lincoln, NE, USA), and the signals were visualized using an Odyssey infrared imaging system (LiCor BioSciences, Lincoln, NE, USA).

2.7. Fluc activity assay

Time-course analysis of the Fluc expression was performed in 24-well tissue culture plates. SK6 cells in 24-well plates were infected with CSFV-N^{pro}Fluc or Shimen at a multiplicity of infection (MOI) of 0.1, at the different time-points post-infection, the supernatant was removed and the virus-infected SK6 cells were washed with cold PBS. The cells in each well (approximately 2.5×10^5 -cells) were lysed with 100 μl of the passive lysis buffer (Promega, Madison, WI, USA), followed by incubation on a shaker for 20 min at 4 °C. The lysate was centrifuged for 5 min at 10,000×*g* and 4 °C. The supernatant was assayed for the Fluc activity. For the siRNA screening assay in 48-well plates (approximately 1.2×10^5 cells) and the neutralization test in 96-well plates (approximately 5×10^4 cells), the cells were lysed with 50 or 30 μl of the passive lysis buffer, respectively. For all assays, the Fluc activity in relative light units (RLU) was measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), according to the manufacturer's instructions.

2.8. RT-PCR

Total RNA was extracted from virus-infected SK6 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I to remove potential genomic DNA. RNA concentration was measured spectrophotometrically at 260/280 nm (Thermo Fisher Scientific, Waltham, MA, USA). Synthesis of cDNA was performed in a 20-μl volume containing 200 ng of total RNA, 20 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (TaKaRa, Dalian, China), 200 μM deoxynucleoside triphosphate mixture (dNTPs) (TaKaRa, Dalian, China), 0.4 μM random primers (TaKaRa, Dalian, China), 0.5 μl of RNase inhibitor (TaKaRa, Dalian, China), and 4 μl of 5× M-MLV reverse transcriptase buffer. The mixture was incubated for 1 h at 42 °C, followed by 15 min at 75 °C. The resulting cDNA was used as template for subsequent PCR with Platinum® Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA) and the primer pair flanking the N^{pro} gene, N^{pro}-F (5′-CAC ATG GAG TTG AAT CAT TTT G-3′)/N^{pro}-R (5′-GAA CAA CTG GTA ACC CAC AAT G-3′). The PCR amplicons were subjected to electrophoresis on a 1% agarose gel and sequenced using standard methods.

2.9. Fluc activity-based neutralization test (CSFV-Fluc-NT)

Fifty swine sera were obtained from pigs immunized with the chimeric vector-based CSFV vaccine rAdV-SFV-E2 and challenged with the Shimen strain, as described previously (Sun et al., 2013). The CSFV-Fluc-NT was performed in 96-well plates (approximately 5×10^4 cells/per well). The sera were heat-inactivated for 30 min at 56 °C, twofold serially diluted from 1:10 to 1:2560, and incubated with CSFV-N^{pro}Fluc at 37 °C for 1 h in 5% CO_2. The virus–antibody mixtures were transferred to the 96-well plates containing the cell monolayers. The luciferase assay was performed after incubation for 48 h at 37 °C. Each serum dilution was tested in duplicate, with appropriate positive and negative controls. The CSFV-specific NAbs titers were expressed as the reciprocal of the highest dilution that resulted in 50% reduction of the Fluc activity. In addition, all sera were tested in parallel by the CSFV Antibody ELISA (IDEXX, Liebefeld-Bern, Switzerland) (positive, ⩾40% blocking; negative, ⩽30% blocking).

2.10. CSFV-specific siRNAs preparation and transfection

Twelve CSFV-specific siRNAs (Table 1) targeting the CSFV N^{pro}, p7, NS5A or NS5B genes (three siRNAs for each gene) were designed based on the genome of the CSFV Shimen strain and a control scramble siRNA (siScr) which has no matches either in the viral or the porcine genome was also included. All siRNAs were synthesized by Shanghai GenePharma (Shanghai, China). All candidate sequences targeting the four genes were screened against the GenBank database using BlastN to avoid homologous sequences in the pig genome.

PK-15 cells at 70% confluence in 48-well plates were transfected with the different siRNAs using X-tremeGene siRNA Transfection Reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. The siRNAs had a final concentration ranging from 50 to 150 nM. After incubation for 24 h, 50 $TCID_{50}$ CSFV-N^{pro}Fluc or Shimen were added to the cells. At 48 hpi, the Fluc activity (CSFV-N^{pro}Fluc) and the viral titers (Shimen) were determined as described above.

2.11. Statistical analysis

Correlations within cohorts were evaluated using the Spearman correlation test. Differences between groups were examined for statistical significance using Student's *t*-test. An unadjusted *P*-value less than 0.05 was considered to be significant.

3. Results

3.1. Generation and characterization of the recombinant CSFV expressing Fluc

Viable virus CSFV-N^{pro}Fluc was readily obtained after the SK6 cells transfected with pBRCISM-N^{pro}Fluc were continuously passaged four times. To define the characteristics of CSFV-N^{pro}Fluc, we compared the growth kinetics of CSFV-N^{pro}Fluc with the parent virus Shimen. The peak virus titer of CSFV-N^{pro}Fluc was 10^5 $TCID_{50}$/ml, approximately 10-fold lower than that of Shimen, at 10^6 -$TCID_{50}$/ml (Fig. 2A). This is consistent with previous observations that insertion of an additional gene at this position had a slight impact on the *in vitro* growth kinetics of CSFV (Li et al., 2013b).

In order to further characterize CSFV-N^{pro}Fluc, the Fluc activity of CSFV-N^{pro}Fluc- or Shimen-infected SK6 cells was measured at different hpi. The Fluc activity of CSFV-N^{pro}Fluc-infected cells at 12 hpi was about 15-fold higher than that of Shimen, indicating that the initiation of the N^{pro} expression occurred before 12 hpi, and the Fluc activity increased to a peak at 60 hpi, approximately 5×10^6-fold higher than that of the control cells infected with Shimen (Fig. 2B). We also detected the N^{pro} expression using Western blot at different time-points post-infection. The N^{pro}-Fluc fusion protein with the predicted molecular weight was readily detected in infected cells at 12 hpi, and the intensity of the immunostained protein was correlative with the Fluc activity at the corresponding hpi (Fig. 2C).

To determine the earliest time when the Fluc activity can be detected, SK6 cells were infected with CSFV-N^{pro}Fluc and the Fluc activity was measured at 0, 1.5, 3 h and then every 1.5 h until 12 hpi. At 4.5 hpi, we observed a twofold higher signal than that of 0 hpi, which was statistically significant ($P = 0.0098$), indicating that the process from uptake of CSFV to initiation of the viral gene expression took less than 4.5 h (Fig. 2D). These results demonstrated that

Table 1
Sequences of siRNAs tested in this study.

Target genes	Names of siRNAs	Sequences of siRNAs (5′–3′)
N^{pro}	siN^{pro}-108	GAGUGAGGUACACCCACAATT
	siN^{pro}-231	GGUUAGCGGGAUAUAUGUATT
	siN^{pro}-367	GGUAGUGACGGAAAGCUUUTT
p7	siP7-55	GACAUCGAGGUCGUAGUAUTT
	siP7-101	GGGAUGAGCCUAUAAAGAATT
	siP7-167	CCAUUACAGUGGCAUUGCUTT
NS5A	siNS5A-239	GCUCAUUUCUCUGCAGAAATT
	siNS5A-503	GCGAGAAACCGAACCACAATT
	siNS5A-734	GGACCAUAAGACCAGCCUUTT
NS5B	siNS5B-285	GCACAACGACUGGAUAAUUTT
	siNS5B-921	GCAGAAGCCAGUAGUUAUATT
	siNS5B-1234	GCAGGCAAUAGCAUGCUAATT
None	siScr	UUCUCCGAACGUGUCACGUTT

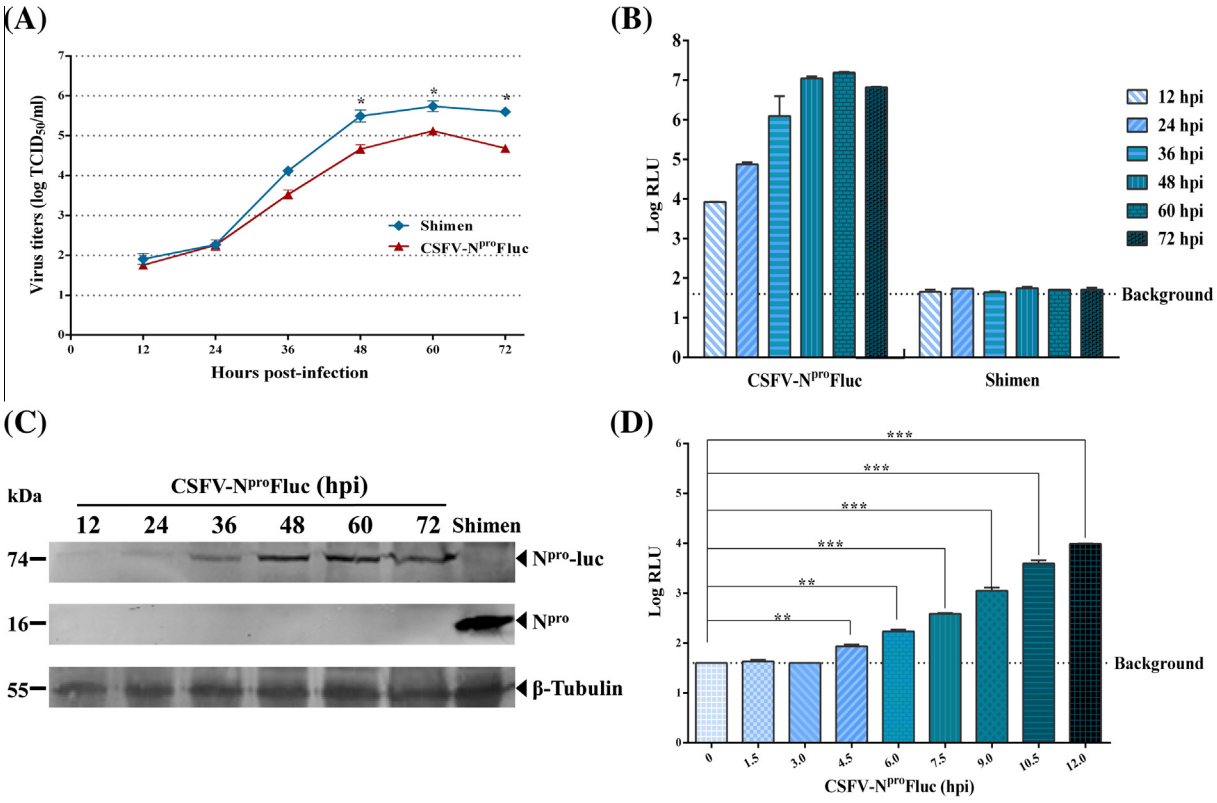

Fig. 2. Characterization of the CSFV-N^{pro}Fluc. (A) Growth kinetics of CSFV-N^{pro}Fluc. SK6 cells were infected with Shimen or CSFV-N^{pro}Fluc at an MOI of 0.1. Viral titers from culture supernatants at indicated time points were determined by the indirect immunofluorescent assay. Each value represents the average of three independent experiments. (B) Time-course analysis of the reporter gene expression. The Fluc activity in relative light units (RLU) was measured in the SK6 cells following infection with the CSFV-N^{pro}Fluc or Shimen at the indicated time points (MOI = 0.1). Data represent three independent experiments and are shown as mean ± standard deviation. (C) Western blot analysis of the N^{pro}-Fluc fusion protein expression. SK6 cells were infected with CSFV-N^{pro}Fluc (MOI = 0.1) at the indicated time points. β-Tubulin was used as a loading control. (D) Initiation time of the N^{pro} expression. The expression of N^{pro}-Fluc was monitored by measuring the Fluc activity in relative light units (RLU) in the SK6 cells infected with CSFV-N^{pro}Fluc at an interval of 1.5 h until 12 h post-infection. Data represent three independent experiments and are shown as mean ± standard deviation, and *P*-values of paired Student's *t*-test are shown ($^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$).

CSFV-N^{pro}-Fluc could be a useful tool for detecting the viral gene expression in the early stage of CSFV infection.

3.2. Stability of the CSFV-N^{pro}Fluc after multiple passages

To examine the stability of the inserted Fluc gene, we passaged the virus in two porcine cell lines (SK6 and PK-15 cells) for 10 times. Briefly, cells in a 25-cm^2 flask were infected with the rescued CSFV-N^{pro}Fluc (defined as P0) at an MOI of 0.1. At 72 hpi, 200 μl of the clarified cell culture supernatants from the passaged virus (P1) were added to näive cells to get passage 2 virus (P2). After 10 rounds of serial passage, the viral RNA was extracted from the supernatant of the infected cells of each passage (P0–P10) and detected the N^{pro}-Fluc fusion gene by RT-PCR. The RT-PCR products with an expected 2151 bp were detected from the supernatant of the infected SK6 cells (Fig. 3A) and PK-15 cells (data not shown),

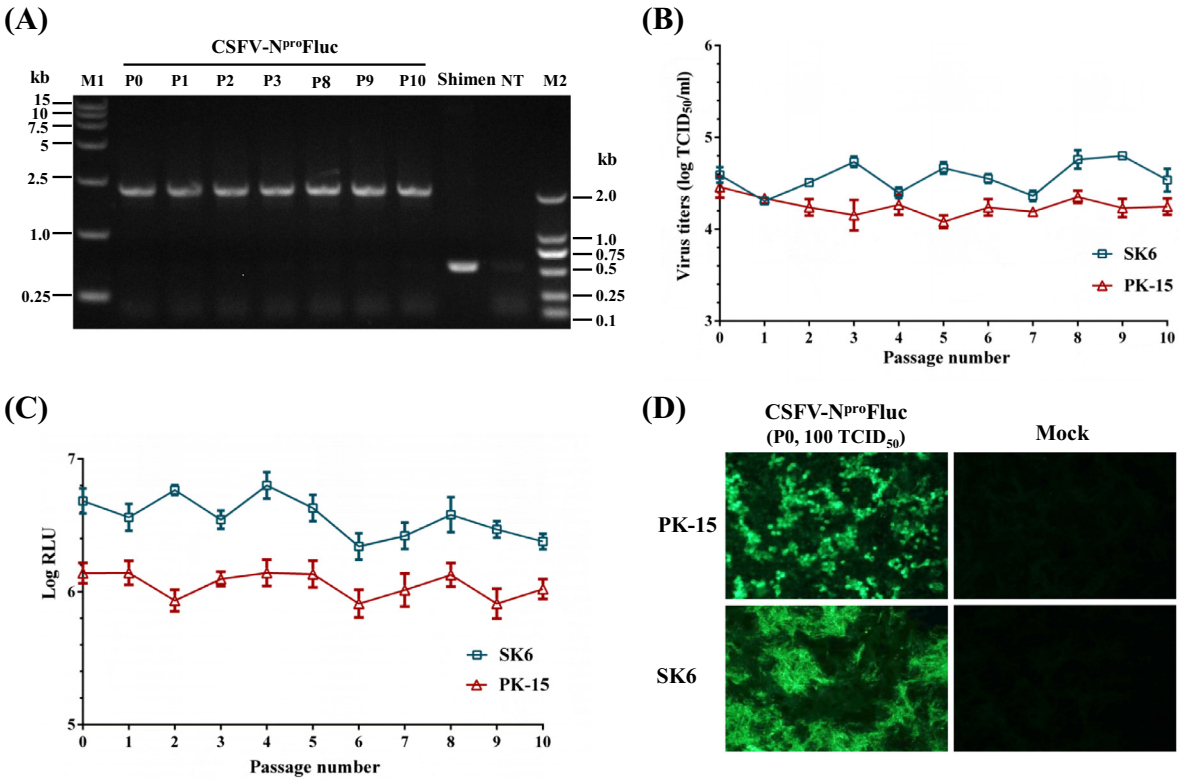

Fig. 3. Stability of the CSFV-N^{pro}Fluc in cell culture. (A) Detection of the Fluc gene during virus passage. Viral RNA was extracted from culture supernatants of different passages in SK6 cells, and RT-PCR was performed with a primer set flanking the N^{pro} protein. The resulting RT-PCR products were resolved by 1% agarose gel electrophoresis. (B) Viral titers of CSFV-N^{pro}Fluc during serial passage. CSFV-N^{pro}Fluc was passaged 10 times in SK6 and PK-15 cells, and the supernatant was collected from the cells infected with CSFV-N^{pro}Fluc of each passage and titrated using the IFA-based viral titration assay. Data represent three independent experiments and are shown as mean ± standard deviation. (C) The Fluc activity of CSFV-N^{pro}Fluc of each passage. SK6 and PK-15 cells were infected with 100 $TCID_{50}$ CSFV-N^{pro}Fluc of each passage in 24-well plates and assayed for the Fluc activity in relative light units (RLU) at 48 h post-infection. Data represent mean values of three independent experiments with error bars representing the standard deviations of the means. (D) Infectivity assay of CSFV-N^{pro}Fluc in two cell lines. SK6 and PK-15 cells were infected with the rescued virus CSFV-N^{pro}Fluc (P0) in 24-well plates at a dose of 100 $TCID_{50}$ and analyzed using the indirect immunofluorescent assay at 48 h post-infection.

and no unexpected mutations were found by sequencing the RT-PCR products, indicating that the Fluc gene remained intact in the genome of CSFV-N^{pro}Fluc.

Moreover, the viral titers of CSFV-N^{pro}Fluc of each passage were determined using IFA. The results showed that the viral titers of the reporter virus passaged in PK-15 cells were about 2 times lower ($10^{4.13}$–$10^{4.45}$ $TCID_{50}$/ml) than those of the virus passaged in SK6 cells ($10^{4.34}$–$10^{4.80}$ $TCID_{50}$/ml) (Fig. 3B). We also infected both cells lines in 24-well plates with each passage at 100 $TCID_{50}$ and measured the Fluc activity at 48 hpi. Our results showed that the Fluc activity of the reporter virus at each passage showed no significant fluctuations and the Fluc activity determined in SK6 cells ($10^{6.34}$–$10^{6.81}$) was about 6 times higher than that of PK-15 cells ($10^{5.91}$–$10^{6.14}$) (Fig. 3C). Taken together, the results demonstrated that the Fluc gene was expressed stably during the 10 passages in cell culture.

3.3. Comparison of CSFV-Fluc-NT and IDEXX-ELISA

The anti-CSFV NAbs titers measured by CSFV-Fluc-NT correlated well with the blocking rates measured by IDEXX-ELISA, with a strong coefficient of correlation (R^2 = 0.9194) (Fig. 4), suggesting that CSFV-N^{pro}Fluc can be used to quantify anti-CSFV NAbs.

3.4. Screening for anti-CSFV siRNAs

To further evaluate the applicability of CSFV-N^{pro}Fluc for antiviral screening, 12 CSFV-specific siRNAs targeting N^{pro}, p7, NS5A or

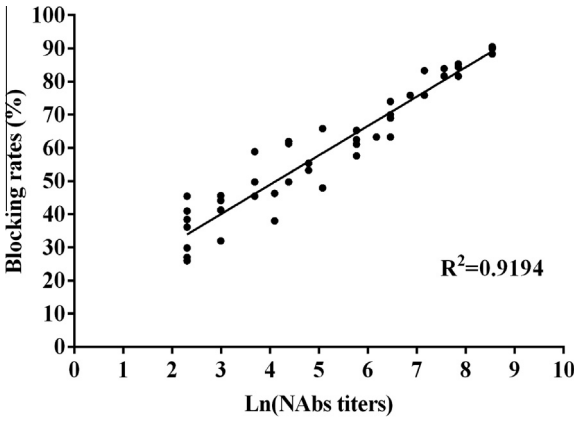

Fig. 4. Correlation between CSFV-Fluc-NT and IDEXX-ELISA. The relationship between the blocking rates and the logarithms of the NAbs titers was analyzed based on the data from 50 serum samples obtained from pigs vaccinated with the CSFV vaccine rAdV-SFV-E2 and challenged with the CSFV Shimen strain. The serum samples, the 1:10 initial dilution of which failed to show 50% reduction of the Fluc activity in relative light units (RLU), were defined as negative.

NS5B genes were assessed. The Fluc activity was significantly decreased in the cells transfected with any of the siRNAs at a concentration of 100 nM, especially siN^{pro}-108 (P = 0.0011), siNS5A-239 (P = 0.0032) or siNS5B-1234 (P = 0.0054) (Fig. 5A).

To compare the two systems (Fluc versus IFA) and verify if these siRNAs display similar inhibitory effects on the wild-type CSFV, we analyzed in parallel the antiviral activity of four siRNAs

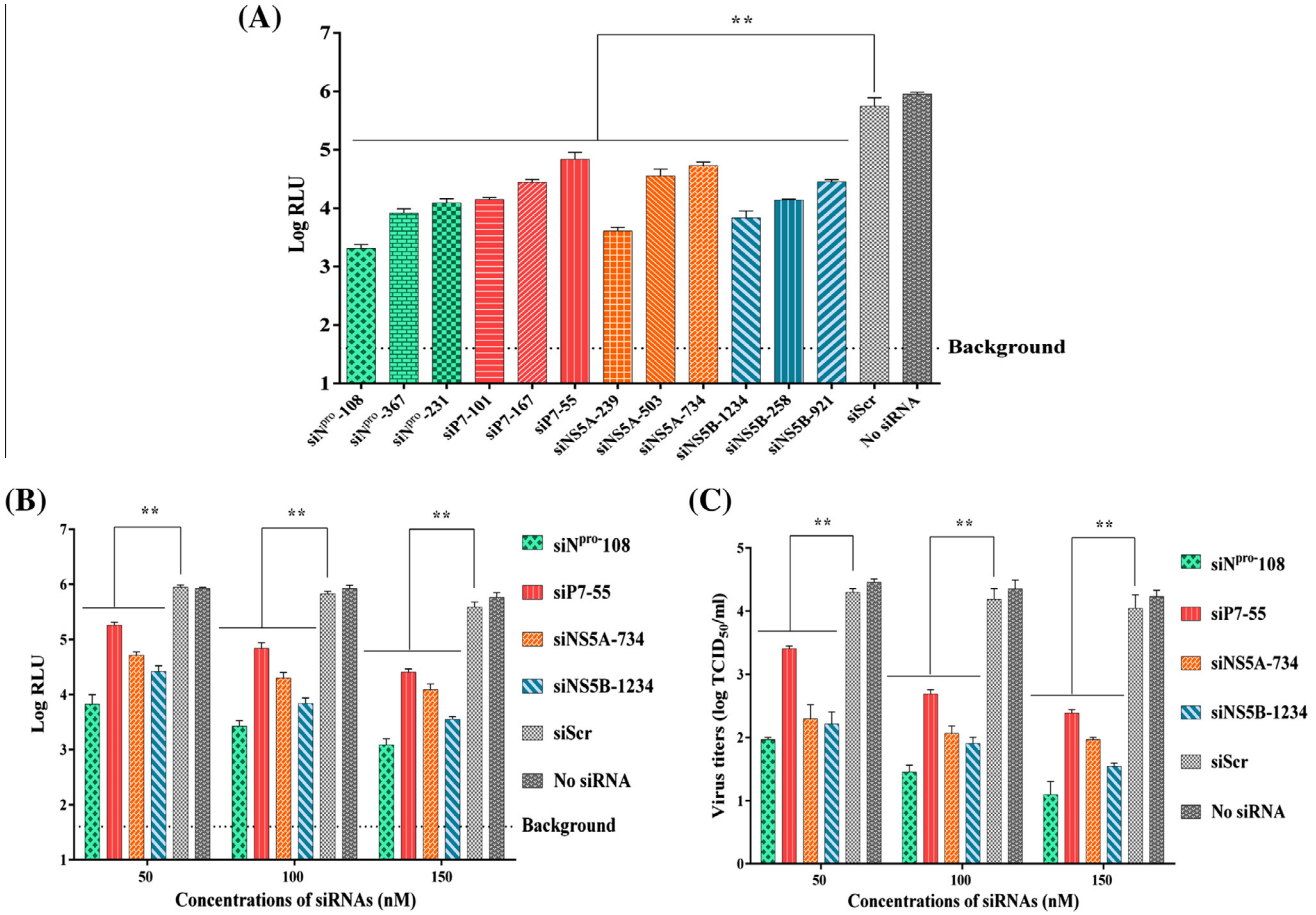

Fig. 5. Antiviral siRNAs screening using CSFV-N^{pro}Fluc or the parent virus Shimen strain. (A) Screening of antiviral siRNAs using CSFV-N^{pro}Fluc. PK-15 cells were transfected with 12 siRNAs followed by infection with 50 $TCID_{50}$ CSFV-N^{pro}Fluc in 48-well plates, and the Fluc activity in relative light units (RLU) was measured at 48 h post-infection. (B) Antiviral activity of different doses of the selected siRNAs against CSFV-N^{pro}Fluc. PK-15 cells were transfected with siRNAs at three different transfection doses followed by infection with 50 $TCID_{50}$ CSFV-N^{pro}Fluc, and the Fluc activity in relative light units (RLU) was determined at 48 h post-infection. (C) Validation of antiviral siRNAs using the CSFV Shimen strain. PK-15 cells were transfected with the selected siRNAs at three different transfection doses followed by infection with 50 $TCID_{50}$ Shimen, viral titers were determined by the indirect immunofluorescent assay at 48 h post-infection and expressed as $TCID_{50}$/ml. Data represent three independent experiments and are shown as mean ± standard deviation, and *P*-values of paired Student's *t*-test are shown (**$P < 0.01$).

(siN^{pro}-108, siP7-55, siNS5A-734 and siNS5B-1234) using the CSFV-N^{pro}Fluc-based Fluc activity assay and the Shimen strain-based IFA viral titration assay with different concentrations (50, 100 and 150 nM) of siRNAs. A significant decrease in the Fluc activity (Fig. 5B) or viral titers (Fig. 5C) was observed in cells transfected with the selected four siRNAs compared with the siScr-treated cells, with 870.9-, 44.3-, 111.3- and 313.3-fold reductions in viral titers for 150 nM siN^{pro}-108, siP7-55, siNS5A-734 and siNS5B-1234, respectively (Fig. 5C), which were comparable to those of the Fluc activity assay, i.e. 332-, 15.8-, 32.45- and 113.2-fold reductions in Fluc activity (Fig. 5B). The results demonstrated the feasibility of using CSFV-N^{pro}Fluc for high-throughput antiviral screening.

4. Discussion

The goal of this study is to generate a Fluc reporter virus for HTS assays. Compared with other fluorescent tags such as EGFP (Li et al., 2013b) or bacterial chloramphenicol acetyltransferase (CAT) (Moser et al., 1998), the Fluc tag has several advantages, e.g., ease to use and high sensitivity. Although the peak viral titer of CSFV-N^{pro}Fluc was lower than that of the parent virus, our results demonstrated that this did not compromise the measurement of the Fluc activity in cell lysates (Fig. 2B and C). Moreover, the Fluc-tagged CSFV-N^{pro}Fluc remained stable after 10 passages in PK-15 or SK6 cells (Fig. 3A–C). Also, we found that CSFV was reproduced more efficiently in SK6 cells than in PK-15 cells as demonstrated by IFA (Fig. 3D), which may explain why the Fluc activity was higher in SK6 cells than that in PK-15 cells (Fig. 3C). N^{pro} is the first protein encoded by the viral ORF which cleaves itself at a cysteine-serine dipeptide site at its C-terminus, thus releasing the N-terminus of viral nucleocapsid (C) protein from the nascent viral polyprotein (Seago et al., 2010). Initiation of N^{pro} synthesis represents the viral gene expression. Our results showed that the Fluc-N^{pro} fusion expression was detectable within 4.5 hpi (Fig. 2D), and this happens to be the first report about the initial time of viral protein synthesis in CSFV-infected cells.

Neutralization assays have been used for many types of studies, such as laboratory diagnosis, vaccine development, epidemiological study, etc. These studies are often intended for a large number of samples. A high-throughput neutralization test based on a reporter respiratory syncytial virus has been established for measurement of NAbs (Fuentes et al., 2013). In this study, we developed a novel Fluc activity-based neutralization assay using CSFV-N^{pro}Fluc and demonstrated that the anti-CSFV NAbs titers measured in CSFV-Fluc-NT correlated well with the blocking rates measured by IDEXX-ELISA (Fig. 4). The CSFV-Fluc-NT offers several significant advantages over the traditional Shimen-NT. First, the

CSFV-Fluc-NT is labor- and time-saving without the incubation and staining procedures, and can be achieved by automated measurement of the luciferase activity rather than depending on the visual inspection scoring for IFA. Moreover, the CSFV-Fluc-NT is applicable to batch analysis since the sample plates can be frozen and stored (after cell lysis) for several days without affecting the read-outs.

Successful RNA interference with inhibition of CSFV replication has already been demonstrated by synthetic siRNAs (Xu et al., 2008) and retroviral vector-mediated siRNAs targeting N^{pro}, NS3, NS4A and NS5B genes (Li et al., 2011). To our knowledge, no siRNAs targeting p7 and NS5A of CSFV have been reported. The CSFV p7 has been recently regarded as a class II viroporin which plays an essential role in the life cycle of pestiviruses and contributes to their pathogenicity (Gladue et al., 2012). The exact function of NS5A in the life cycle of CSFV remains poorly understood. NS5A may regulate the viral RNA replication by binding to NS5B and 3′-UTR (Chen et al., 2012; Sheng et al., 2012). Here, we assessed 12 siRNAs against the CSFV N^{pro}, p7, NS5A or NS5B genes using CSFV-N^{pro}Fluc with three siRNAs being able to efficiently inhibit the CSFVN^{pro}Fluc replication. We also confirmed the inhibitory effects of four siRNAs by IFA using the parent virus. Compared to the IFA-based viral titration assay, the Fluc activity assay has several advantages. First, it can be used in HTS assays in 48 or 96-well plates, which means screening of antivirals from libraries containing several hundred compounds is feasible. Second, it is time-saving. We can directly evaluate the inhibitory effects of the antivirals in the plates, without multiple steps for IFA, which require extra 72 h. Third, it is more sensitive, for it can detect a signal between 10^2 and 10^3 RLU. By contrast, such a low level of CSFV can hardly be titrated by the traditional IFA-based viral titration assay. Therefore, CSFV-N^{pro}Fluc can be used for high-throughput antiviral screening with higher efficiency. To our knowledge, this is the first report that a luciferase reporter CSFV was constructed and used for antiviral testing. The reporter virus will contribute to future work focused on high-throughput screening of antivirals from compound libraries.

In summary, we generated a recombinant CSFV stably expressing the Fluc gene, which allows rapid and sensitive quantification of viral replication and screening of antivirals against CSFV.

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