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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,

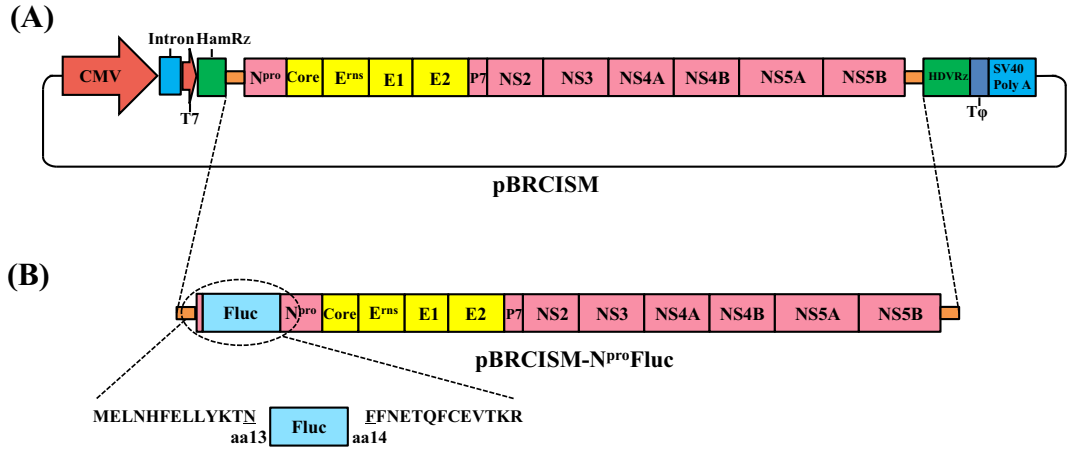

Fig. 1. Schematic representation of the cDNA clones pBRCISM (A) and pBRCISM-N^{pro}Fluc (B). The infectious cDNA clone of the CSFV Shimen strain was used as the backbone for construction of the cDNA clone of CSFV harboring the Fluc gene, which was introduced into the N^{pro} gene by overlapping PCR as 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.)