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A dark-to-bright reporter cell for classical swine fever virus infection

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ABSTRACT

Current methods to quantitate classical swine fever virus (CSFV) infectivity in cell culture are time-consuming and labor-intensive. This study described the generation of a dark-to-bright fluorescent reporter cells to facilitate *in vitro* studies of CSFV infection and replication. This assay was based on a novel reporter cell stably expressing the enhanced green fluorescent protein (EGFP) fused in-frame to a quenching peptide via a special recognition sequence of the CSFV NS3 protease. Chromophore maturation of EGFP can be prevented by quenching peptide until the quenching peptide was specifically cleaved by NS3 protease during CSFV infection, making it a dark-to-bright reporter of CSFV infection. The result demonstrated that the CSFV-infected cells were clearly distinguishable from mock-infected cells and cells infected with other viruses. There was a strong correlation between the fluorescence intensity and viral RNA replication in CSFV-infected cells. The cell enabled rapid and sensitive detection of CSFV infection and viral replication in cell culture. The best time to examine the fluorescence in CSFV-infected cells was at 48 h post-inoculation. These data suggested that the cells can be used as a reporter cell in CSFV infection assays. This reporter cell provides a sensitive method for the detection and isolation of CSFV and it will be useful for the screening of antiviral drugs or neutralizing antibody assays.

1. Introduction

Classical swine fever virus (CSFV) is a small, enveloped, plus-strand RNA virus and has been found to be infectious only for pigs (van Gennip et al., 2004). CSFV genome contains a 5′-untranslated region (5′UTR), 3′-untranslated region (3′UTR) and a single large open reading frame (ORF) (Dreier et al., 2007; Greiser-Wilke et al., 2007). The ORF is translated into a polyprotein, which is processed by viral and cellular proteases into the individual structural and nonstructural proteins (Sheng et al., 2007; Wen et al., 2009). Nonstructural protein 3 (NS3) is a multifunctional protein possessing serine protease, RNA helicase and nucleoside triphosphatase (NTPase) activities located in two functionally distinct domains. The N-terminal one-third of NS3 primarily serves as a protease to process the viral polyprotein, which is required for processing at nonstructural protein sites 3/4A, 4A/4B, 4B/5A and 5A/5B (Xu et al., 1997; Wen et al., 2009).

Although the traditional diagnostic tools such as VNT (virus neutralization test) or virus isolation have a long history and continue to be the "gold standards" for serological and virological investigations, almost no published information on standardization and validation is available (Greiser-Wilke et al., 2007). Nevertheless, they have proven to be invaluable tools for the diagnosis of CSF (Greiser-Wilke et al., 2007). Molecular technologies, such as RT-PCR, real-time quantitative PCR and RT-LAMP (reverse transcription loop-mediated isothermal amplification), could be used as the first choice for detection of CSFV due to the high sensitivity (Backer et al., 2011; Chen et al., 2010; Leifer et al., 2011; Wang et al., 2010; Wen et al., 2010; Zhang et al., 2010), while they were vulnerable to false positive results arising from sample to sample contaminations or from other contaminated sources (Wang et al., 2010). Furthermore, antigen ELISA-related assays, such as colloidal gold immunochromatographic assay and antigen-capture ELISA, were not widely used due to the low sensitivity

Abbreviations: CSFV, classical swine fever virus; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; hpi, hours post-inoculation; NS, nonstructural protein; ORF, open reading frame; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PCV2, porcine circovirus; PHEV, porcine hemaegglutinating encephalomyelitis virus; PK-15, porcine kidney cell line-15; PRRSV, porcine reproductive and respiratory syndrome virus; PRV, pseudorabies virus; RNA, ribonucleic acid; real-time RT-PCR, real-time reverse transcription PCR; RT-PCR, reverse transcription PCR; UTR, untranslated region; $TCID_{50}$, 50% tissue culture infective dose.

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(Kaden et al., 1999; Dewulf et al., 2004; Reither et al., 2009; Wang et al., 2010). Another disadvantage was that both ELISA-related assay and nucleotide amplification assay cannot differentiate live virus from dead virus.

Transgenic cell line provides a simple and virus-specific detection system (Olivo, 1996). The basic strategy is to stably introduce genetic elements into a cell in such a way that when a particular virus enters in the cell, a virus-specific event that results in the production of an easily measurable enzyme is triggered (Olivo, 1996; Leland and Ginocchio, 2007). Cell lines expressing reporter genes inducible upon viral infection have shown to be both sensitive and specific (Olivo, 1996; Lee et al., 2004; Lutz et al., 2005; Wang et al., 2006; Ueno et al., 2006; Wu et al., 2007; Fukui et al., 2008; Li et al., 2009; Iro et al., 2009; Hossain et al., 2010; Levy et al., 2010). These reporter cells exploit the specificity of viral infection in combination with the extreme sensitivity of reporter proteins such as luciferase, chloramphenicol acetyltransferase (CAT), LacZ (β-D-galactosidase), green fluorescence protein (GFP) or secreted alkaline phosphatase (SEAP). These approaches expedite simultaneous detection as well as amplification of virus present in the clinical specimen, providing a live virus stock for further analyses. However, reporter cell for CSFV infection has not been reported.

It was reported that fluorescent proteins can be activated from a silent dark state to a bright fluorescent state directly by the activity of an enzyme (Mahajan et al., 1999; Nicholls et al., 2011; Nicholls and Hardy, 2013). Chromophore maturation of GFP is prevented because the GFP protein does not fold into the correct pattern until the quenching peptide has been specifically cleaved by certain enzyme (Nicholls and Hardy, 2013). The aim of this study was to develop a cell-based EGFP reporter system, in which fluorescence is fully quenched by a short peptide. However, the fluorescence can be fully restored during CSFV infection due to catalytic removal of the quenching peptide by CSFV NS3 protease, making it a dark-to-bright reporter of CSFV infection. This cell-based reporter system provides a sensitive method for the detection and isolation of CSFV and it is also useful for the screening of antiviral drugs or neutralizing antibody assays.

2. Materials and methods

2.1. Reagents, cells and virus

Restriction enzymes, T4 DNA ligase and TurboFect™ *in vitro* transfection reagent were purchased from Fermentas (Fisher Scientific, PA, USA). TIANamp Virus DNA/RNA Kit, TIANamp genomic DNA kit, 2 × *Taq* plus mastermix, pGM-T, DNA Marker 2000 and TRIzol-A$^+$ reagent were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). DNA marker 3 was purchased from Bioteke Corporation (Beijing, China). BioRT cDNA first strand synthesis kit and BioEasy SYBR Green I real-time PCR kit was purchased from Bioer (Hangzhou, China). Primers used in this study (Supplemental Table 1) were synthesized by Genscript Biotech Co., Ltd. (Nanjing, China).

Porcine kidney cell line-15 (PK-15) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (PAA, GE Healthcare Bio-Sciences Corp, NJ, USA) and incubated at 37 °C in an atmosphere of 5% CO_2.

CSFV (strain Shimen) obtained from the Institute of Veterinary Drug Control (Beijing, China) was used in this study and the positive anti-CSFV serum was kindly provided by Dr. Changchun Tu (Academy of Military Medical Sciences, Changchun, China). Porcine circovirus (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV) and porcine hemaegglutinating encephalomyelitis virus (PHEV) were stored in our lab.

2.2. Plasmids construction

To generate a cell-based GFP expression vector specific recognized by CSFV, a DNA fragment (*Bgl*II-linker-quench-*Bam*HI) was synthesized. The linker sequence can be specially recognized and cleaved by CSFV NS3 serine protease. The quench sequence was a 27-amino acid peptide derived from the tetrameric proton channel domain of influenza M2 protein according to the method described by Nicholls (Nicholls et al., 2011). The fragment was synthesized and inserted into pEGFP-C1 (BD Biosciences Clontech, CA, USA) by replacing the fragment using *Bgl*II and *Bam*HI in pEGFP-C1, resulting in pEGFP-linker-quench (Fig. 1). The plasmid was linearized by *Ase*I and stably transfected into PK-15 cells.

2.3. Generation of stable cell line

To establish a stable cell line, PK-15 cells were seeded in a 60-mm dish at a density of 7×10^5 cells per dish. On the following day, the cells were transfected with 5 μg of linearized plasmid using TurboFect™ *in vitro* transfection reagent according to the manufacturer's instructions. At 24 h after transfection, cells were passaged at 1:36 dilution into new 6-well plates and maintained in fresh DMEM supplemented with 2% fetal bovine serum and 1200 ng/mL G418 (Amresco, OH, USA) for two weeks. The medium was replaced every three days. Stably-transfected cells were trypsinized with 0.5% trypsin and maintained on the same selection medium in separate 6-well dishes for further study. The stable transfection to the cells was identified and confirmed by PCR and fluorescence microscopy (Eclipse TE2000-V, Nikon Imaging, Japan). The cells stably transfected with pEGFP-linker-quench was designated as PK15-CSFV-GFP.

2.4. Virus infection

PK15-CSFV-GFP cells were cultured in 24-well plate and incubated overnight. At 70–80% confluency, the cells were infected with CSFV or other porcine viruses (PCV2, PRRSV, PRV and PHEV) at 5 $TCID_{50}$, respectively. At 2 h post-inoculation (hpi), the medium was removed and the cells were cultured in fresh DMEM supplemented with 2% fetal bovine serum. Fluorescence emitted by the cells was observed using a fluorescence microscope Eclipse

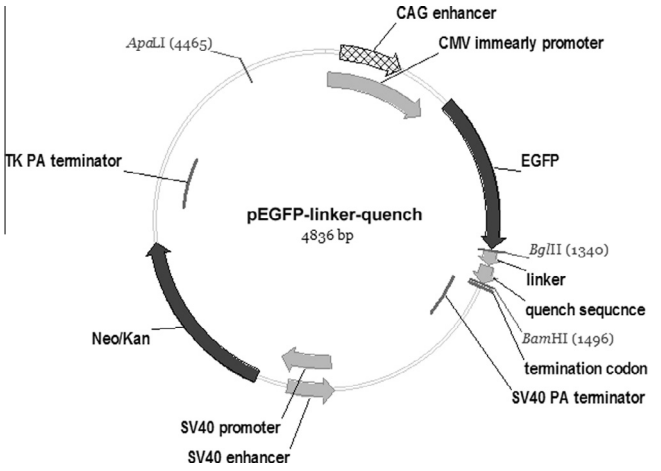

Fig. 1. Schematic diagram of fusion protein construct. DNA fragment (*Bgl* II-linker-quench-*BamH* I) was synthesized and inserted into pEGFP-C1 by replacing the fragment in pEGFP-C1 using *Bgl*II and *Bam*HI, resulting in pEGFP-linker-quench. The construct pEGFP-linker-quench contained the linker sequence and quench sequence. The linker sequence can be specially recognized and cleaved by CSFV NS3 serine protease. The quench sequence was a 27-amino acid peptide derived from the tetrameric proton channel domain of influenza M2 protein.