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Clover-tagged porcine reproductive and respiratory syndrome virus infectious clones for rapid detection of virus neutralizing antibodies

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS), caused by the PRRS virus (PRRSV), is a widespread disease that affects domestic pigs of all ages. Accurate and rapid detection of PRRSV specific neutralizing antibodies levels in a pig herd is beneficial for the evaluation of the herd's immunity to combat the specific viral infection. However, the current methods for viral detection, including fluorescent focus neutralization (FFN) and cytopathic effect (CPE) reduction neutralizing assays, are subjective and time-consuming. Therefore, a Clover-tagged PRRSV virus neutralization assay were developed that instrumentally measures the fluorescence signal of Clover stably expressing by a PRRSV infectious clone for at least 10 passages. Herein, the results showed that the proposed Clover-tagged PRRSV neutralization assay is reliable using instrumental measurements of the fluorescence signal of Clover and allows for rapid detection of neutralizing antibodies against PRRSV. The assay was evaluated by testing swine sera from experimental and field samples, and comparisons were made with the traditional FFN and CPE reduction assays. These results suggest that the Clover-tagged PRRSV infectious clone offers a fast and reliable testing method for neutralizing antibodies and could permit high-throughput screening of new antiviral agents.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a widespread disease that affects domestic pigs of all ages (Collins et al., 1992; Wensvoort et al., 1991). The causative agent is the PRRS virus (PRRSV), a member of the *Arteriviridae* family of the order *Nidovirales*, which is divided into Type 1 (European) and Type 2 (North American) species. In 2006, there was a highly pathogenic PRRS, caused by highly pathogenic PRRSV Type 2 (HP-PRRSV), outbreak in China, which was characterized by high grade fever with increased mortality in pigs of all ages (Tian et al., 2007). The high degree of genetic variation of the virus restricts the generation of cross-protective immunity between isolates, resulting in limited protection offered by commercial vaccines (Popescu et al., 2017).

Virus-specific neutralizing antibodies are a critical part of the immune armory in virus defense and are part of the mechanism for effective vaccination. PRRSV is highly heterogenic, and different isolates are various in their sensitivity to neutralization (Martinez-Lobo et al., 2011). The fast detection of serum neutralization antibodies would provide useful information for the veterinarians to determine the protective immunity elicited by vaccinations. Traditional methods include virus serum neutralization assays, which are based on cytopathic effect (CPE) recoding and calculations after incubation for 5 days (Kim et al., 2007b), and the fluorescent focus neutralization (FFN) assay, which requires a time-consuming process of indirect immunofluorescent assay (IFA) and manual foci counting (Wu et al., 2001).

Virus reverse genetics technologies have helped to understand the biology of RNA viruses (Almazan et al., 2006; Gonzalez et al., 2002; Kwon et al., 2011; Park et al., 2012; Racaniello and Baltimore, 1981; St-Jean et al., 2006; Zhang et al., 2012). Several studies have reported numerous infectious clones of distinct PRRSV strains (Fang et al., 2006; Nielsen et al., 2003; Truong et al., 2004; Wang et al., 2013; Zhang et al., 2011). For instance, bacterial artificial chromosome (BAC) systems have effectively been applied to both DNA (Borst et al., 1999; Kanda et al., 2004; Messerle et al., 1997; Suter et al., 1999) and RNA viruses (Almazan et al., 2006, 2000; Wang et al., 2013) due to their large fragments capacity, high replication fidelity, and straightforward manipulation procedures.

Fluorescent protein insertion is a common strategy for labeling recombinant viruses. Clover, derived from a wild-type GFP with S65G to

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maintain a deprotonated chromophore, T203H for higher brightness, and Q69A for higher photostability mutations, is one of the brightest green fluorescent proteins and has thus been applied in the ratiometric fluorescence resonance energy transfer (FRET) pair assay (Lam et al., 2012).

In this study, a HP-PRRSV infectious clone was generated that expresses the Clover fluorescence protein for fast and reliable virus neutralization assay. The Clover gene was inserted between the N gene and 3′UTR region of the PRRSV viral genome and relied on the PRRSV transcription regulatory sequence 2 (TRS2) for its transcription. The recombinant PPRSV rSD16/TRS2/Clover was rescued in MARC-145 cells, and the expressions of fluorescent proteins remained stable for at least 10 passages. Based on PRRSV rescue results, the fluorescent signal of Clover expressed in the cell displayed brighter signals than that of EGFP. In the virus neutralization assay using rSD16/TRS2/Clover, the results from the method based on instrumental measurement of Clover was faster and more reliable than the FFN assay and more time-saving than the CPE reduction assay.

2. Materials and methods

2.1. Viruses, cell lines and serum samples

The PRRSV Type 2 parental strain SD16 (GenBank: JX087437) used in this study was isolated by our lab (Wang et al., 2013). MARC-145 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, USA) at 37 °C in a 5% CO_2 incubator. The PRRSV-negative serum was confirmed by ELISA assay with IDEXX PRRS X3 Ab Test (IDEXX, USA) and RT-PCR. Fifteen pig serum samples were collected from pigs experimentally infected with PRRSV after 4 weeks. Eighty-one clinical pig serum samples were collected from pigs ages ranged 35–168 days from pig herds (age of 35–168 days) determined to be PRRSV-positive by a RT-PCR test. The primers used in the PCR analysis were 5′-ATACTGTGCGCCTGATC CGC-3′ (located in the 3′ end of the N protein) and 5′- TCGCCAATTA AACTTTACCCCCACA-3′ (located in the 5′ end of the 3′ UTR region).

2.2. Plasmid construction

The Clover gene (GenBank: JX489388) was synthesized, and its expression was driven by the transcription-regulating sequence of gene GP2 (TRS2). The vector-containing *Asc*I-*Rsr*II regain was constructed with *Nde*I and *Mlu*I sites between the N gene and 3′ UTR region of the viral genome and denoted pEasy-SD16-AR-NM. The Clover gene was flanked by the *Nde*I-TRS2 and *Mlu*I sites by specific primers (*Nde*I-TRS2-Clover-F: 5′- CATATG*TTGAACCAACTTTAGGCCTGAATTGAA*ATGGTG AGCAAGGGCGAGGAGC-3′, Clover-*Mlu*I-R: 5′- ACGCGTCGTCACTTGT ACAGCTCGTCCATCCCA-3′) and then inserted into pEasy-SD16-AR-NM and further named pEasy-SD16-AR-TRS2-Clover. After digestion with *Asc*I and *Rsr*II, the fragment with the TRS2-Clover sequence was inserted into plasmid pBAC-SD16, which was denoted pBAC-SD16-TRS2-Clover (Fig. 1A). Plasmid pBAC-SD16-TRS2-EGFP was constructed as described above except the use of the specific primers.

2.3. Transfection and rescue of recombinant viruses

For recombinant PRRSV rescue, 80% confluent MARC-145 cells cultured in 6-well plates were separately transfected with the plasmid pBAC-SD16-TRS2-Clover using the Attractene Transfection Reagent (QIAGEN, Germany). Plasmid pBAC-SD16 with the entire original genome of SD16 was used as the positive control. After 72 h, the fluorescence images were captured, and the culture supernatants were used to infect MARC-145 cells to propagate the rescued virus. The rescued viruses were designated as rSD16/TRS2/Clover. Transfection of the plasmid pBAC-SD16-TRS2-EGFP is performed using the same method as described above. The rescued viruses were designated as rSD16/TRS2/EGFP.

2.4. Recombinant PRRSV propagation and growth curve determination

The growth curves of rSD16/TRS2/Clover (the 6th passage) and parental virus SD16 were determined. After 1 h of virus absorption, MARC-145 cells were washed three times with phosphate-buffered saline (PBS) and incubated in DMEM with 3% fetal calf serum at 37 °C and 5% CO_2. The supernatants were collected in a series of time points at 12, 24, 36, 48, 60, 72, and 84 h post infection (hpi), and viral titers were tested using the Reed-Muench method.

2.5. Virus titers and clover gene in different virus passages

For virus titers detection in different virus passages (2nd, 4th, 6th, 8th, and 10th passages), the culture supernatants were collected at 60 hpi, and viral titers were tested as described above. For Clover gene detection in different virus passages, RNA extraction and reverse transcription of totally mRNA in culture supernatant were processed, Clover genes were amplified using the primers (N-F: 5′-CAGGGAGGATAAGT TACACTG-3′, 3UTR-R: 5′-CGCCAATTAAACTTTACCCCCAC-3′), and the nucleic acid sequence of PCR fragments were verified by sequencing.

2.6. Quantification of clover and EGFP fluorescence

MARC-145 cells grown in 96-well plates were infected with rSD16/TRS2/Clover or rSD16/TRS2/EGFP at an MOI of 0.01 (in triplicate). When the cells showed 100% CPE, the cells are washed with PBS (200 μL/well/time, twice), and then 50 μL PBS was added to each well. The green fluorescence signal of each well was read on a multi-detector microplate reader VICTOR™ X5 (PerkinElmer, USA) at Ex/Em = 485/535 nm. The quantities of Clover and EGFP were determined by comparing their fluorescence values using mock-infected cells as controls.

2.7. Imaging fluorescent protein expressions and live cell recoding

For virus fluorescence imaging of different passages, MARC-145 cells were grown in a 35-mm cell culture dish to a density of 80% confluence and were then infected with rSD16/TRS2/Clover (MOI: 0.1). After infection for 1 h, the medium was removed and replaced with 2 mL pre-warmed DMEM containing 3% fetal calf serum. The dish was placed in an incubator at 37 °C with 5% CO_2. The fluorescence images of infected cells were captured using an inverted microscope (Leica DMI6000B, Leica, Germany) at 36 hpi.

For live cell imaging, MARC-145 cells were infected with rSD16/TRS2/Clover (the 6^{th} passage, MOI of 0.1). When fluorescence appeared, the dish with the virus-infected cells was placed in the heating insert P (PeCon GmbH, Germany) covered by an incubator S-2 (PeCon GmbH, Germany) at 37 °C incubation (Tempcontrol 37-2, PeCon GmbH, Germany) and supplied with 5% CO_2 (CTR-Controller 3700, PeCon GmbH, Germany). Clover signals were captured every 3 min for a period of 7 h by an inverted fluorescence microscope (Leica DMI6000B, Germany). Images were processed into a movie of 10 frames/s using QuickTime Pro.

2.8. Fluorescence-based virus neutralization assay based on clover

Serum samples were first incubated for 30 min at 56 °C and were serially two-fold diluted in DMEM. Each serum dilution (50 μL) was mixed with an equal volume of rSD16/TRS2/Clover (the 6^{th} passage, 100 TCID50/well) and incubated for 1 h at 37 °C. MARC-145 cells monolayers, prepared in 96-well plates (1×10^4 cells/well), were infected with 100 μL/well (in triplicate) of the serum/virus mixture for 1 h at 37 °C. The inoculation medium was discarded and replaced by DMEM supplemented with 3% FBS, then the 96-well plates were incubated at 37 °C and 5% CO_2. At 48 h post-inoculation, green