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Application of real-time quantitative polymerase chain reaction to monitoring infection of classic swine fever virus and determining optimal harvest time in large-scale production

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ABSTRACT

Due to the non-cytopathogenic replication of classical swine fever virus (CSFV) in cell culture, large-scale production of CSFV using bioreactor system remains the problem of monitoring the time of maximum virus production for optimal harvest. In this study, we proposed the application of real-time quantitative PCR assay to monitoring the progress of CSFV infection and yield determination in large scale. The region of *NS5B* of CSFV responsible for CSFV genome replication was used for the designation of primers and probe. Viral titers determined by the real-time quantitative PCR assay were compared with the conventional cell-culture based method of immunofluorescent staining. Results from large scale production show that a similar profile of CSFV production was successfully outlined by real-time quantitative PCR and virus yields were comparable to the results from immunofluorescent staining assay. By using this method, an optimal harvesting time of the production could be rapidly and precisely determined leading to an improvement in virus harvest.

1. Introduction

For efficient virus production in a bioreactor, it is essential to monitor the entire process to predict the time of maximum virus yield, and, thus, the optimal harvest time, to maximize the bioreactor yield. Studies have proposed various methods for monitoring a viral infection process and protein production, including measurements of relative permittivity [1], oxygen uptake rate [2,3], intracellular ATP [4], cell viability [5], and changes in cell diameter [6,7]. However, these methods are primarily applied to infection processes that cause reduced cell viability and increased levels of virus production.

During a persistent infection process, referred to as noncytopathogenic replication, viral particles are continuously released into the medium without causing any evident morphological changes or functional damage to infected cells [8]. During this type of infection, it is not possible to phenotypically distinguish the infected and non-infected cells or, thus, monitor viral infection or production. Infection with the classical swine fever virus (CSFV) provides a typical example of this type of infection.

The CSFV is an enveloped positive-strand RNA virus classified in the *Pestivirus* genus, which is in the Flaviviridae family. The CSFV is highly contagious, with a nearly worldwide distribution, causing disease in pigs and considerable economic implications [9,10]. Routine vaccination against the CSFV is the most common method for preventing and controlling CSFV infection in Taiwan and most other endemic countries [11]. An effective live attenuated vaccine against the CSFV derived from the spleen tissue of the rabbit, has been developed and provides complete clinical and virological protection [11,12]. However, the production process for this vaccine has yield limitations because of difficulties in obtaining large numbers of standardized rabbits, high labor costs, and the risk of contaminants and other substances of the animal tissue present in the products. Although studies have developed a safe and potent live attenuated CSFV vaccine using porcine kidney cell lines [13,14], large-scale production development for a commercial cell-based CSFV vaccine is hampered because of problems in determining the viral titer for harvest. This problem is caused by persistent infection of the porcine kidney cells by the CSFV and noncytopathogenic CSFV replication without recognizable effects in cell culture [15]. Previous studies have used the immunofluorescence method, immunoperoxidase staining, the exaltation of Newcastle disease (END) virus method, and the interference method for CSFV titration for determining the optimal virus titer for harvest [9];

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however, these methods require the use of antibodies and are time-consuming, requiring at least 4 working days for completion [16]. Therefore, the development of an effective method for determining the optimal time and overall production of the virus harvest during the vaccine manufacturing process and the quality control of live virus vaccines remain a research priority.

In this study, we applied the TaqMan probe quantitative polymerase chain reaction for detecting the virus yield and monitoring the CFSV infection in large-scale virus production. The real-time quantitative polymerase chain reaction (Q-RT-PCR) has gained worldwide acceptance for CFSV diagnosis because of rapid detection and high sensitivity; however, its application for monitoring mass virus production is not well described. First, we validated the Q-RT-PCR assay for use in CSFV production, and then evaluated its effectiveness for monitoring vaccine production. We used the NS5B region encoding an RNA-dependent RNA polymerase responsible for CSFV genome replication, for primers and probe designation, and a novel bioreactor, containing two separate liquid and gas stages, for CFSV vaccine production, as described previously [17]. We then used the Q-RT-PCR to evaluate the progress of the CFSV infection in the bioreactor, determined the optimal harvest time, and compared our results with those obtained using the conventional cell culture-based method, immunofluorescent staining (IM).

2. Materials and methods

2.1. Cell line, virus, and vaccine

The porcine kidney cell line, PK15, was provided by the Animal Technology Institute of Taiwan (ATIT; Chunan, Miaoli, Taiwan) and maintained in an Eagle's minimal essential medium (MEM; Gibco, Invitrogen Corp., Carlsbad, CA) supplemented with a 5% cosmic calf serum (Gibco). A laboratory-adapted CSFV strain, Fy-HCV, derived from a lapinized Philippines coronel strain (LPC), was propagated in the PK15 cells in the MEM medium supplemented with the 5% CCS. A cell culture-based CSFV vaccine (LPC-PRK strain, 10 doses/vial, Formosa Biomedical Inc., Taiwan, ROC) was used as the standards for titration in the Q-RT-PCR.

2.2. Large-scale production of the CSFV

A laboratory cell-cultivation system was used. The system comprised two 2–5-L tanks connected by a pipe, containing an operating volume of 1.5–4 L of medium. The system was operated in a sterilized 5% CO_2 incubator at 37 °C, as described previously [17]. The cells were inoculated at 7.5×10^5 cells/mL and were infected with CSFV after a period of cultivation. Carrier strips were collected daily and treated with a 0.25% trypsin solution for cell counts. At a particular interval during the virus production step, samples were collected and the viral titer was determined immediately using the Q-RT-PCR. When a 1×10^6 $FAID_{50}$/mL sample had been obtained, the virus suspension was collected and replaced by a new medium. Virus production at each collection time point was then re-evaluated using IM.

2.3. Determination of the viral titer by using IM

For virus titration, the supernatants from cultures were serially titrated with a medium containing a 5% CCS serum as described previously [17]. The cells were examined using a fluorescent microscope, and the titers of the CSFV (in $FAID_{50}$/mL) were determined according to the methods of Reed and Muench, and expressed as the reciprocal of the highest dilution at which the PK-15 cells were infected in 50% of the culture wells.

2.4. Q-RT-PCR

Viral RNA was extracted by adding 750 mL of a TRIzol® reagent (Invitrogen, USA) and 400 μL chloroform (Merck, Germany) to 250 μL of the sample material. Following centrifugation at 8000 × *g* for 15 min at 4 °C, the aqueous phase was transferred to a new tube containing 1 mL isopropanol (Merck, Germany) and incubated for 10 min at room temperature. After centrifugation for 20 min at 4 °C and washing with 500 μL of cold 75% ethanol, the pellets were air-dried, resuspended in 25 μL of diethypyrocarbonate (DEPC, Sigma, USA) water, and stored at −80 °C.

Reverse transcription was performed using a reverse transcription system kit (Promega, USA) according to the manufacturer's instructions. A final volume of 20 μL containing 5 μL of the RNA solutions was incubated at 42 °C for 90 min, 95 °C for 5 min, and 4 °C for 5 min for cDNA synthesis.

The complete genome of the CSFV (accession number AY663656) was used to design a pair of primers and a probe for the Q-RT-PCR: forward, CSFNS5BF (5′-CACACCCTGCAAGGAAGACA-3′); reverse, CSFNS5BR (5′-CAACATGGTGTTAAGGAGGCT-3′); and probe (5′-ACAGATCAACAACTTTCA-3′). The cycle times were the predenaturation and activation of AmpliTaq Gold® DNA polymerase at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s, and annealing and extension at 60 °C for 60 s.

2.5. Specificity, sensitivity, reproducibility, and viral determination effectiveness of the Q-RT-PCR

To evaluate the sensitivity and specificity of the Q-RT-PCR, serial 10-fold dilutions of a known titer of virus solution were assessed using the Q-RT-PCR and IM. To ensure reproducibility, the coefficients of variation (CVs) for Ct values within runs (intra-assay variability) and between-assay types (inter-assay variability) were also determined.

To determine the CSFV titer using the Q-RT-PCR, a lyophilized commercial cell culture-based CSFV vaccine with a viral titer of $1 \times 10^{6.5}$ $FAID_{50}$/mL was used as a standard. The virus solution was serially titrated in 2-fold dilutions prior to viral RNA extraction. For each dilution, a final amount of RNA (an 80th of the initial amount) was used for the Q-RT-PCR, generating a virus number ranging from 12,500 to 195 $FAID_{50}$/per reaction. After each individual run, the equation of the standard curve was calculated using a regression analysis of the Ct values (y) versus the virus number (x), in which y = Ct values and $x = \log_2$ $FAID_{50}$/per reaction. The viral titer was calculated using $FAID_{50}/mL = 2^x \times 80$ (dilution factor). For each individual test, a standard curve was established and a positive control with a known titer was assayed simultaneously. The titer of the positive control was then evaluated using the standard curve to ensure the reproducibility of each assay performance. Each assay was performed on different days using a freshly diluted virus solution.

2.6. Detection of live virus particles

Known particle numbers of the live CSFV solutions were inactivated through incubation at 120 °C for 40 min and mixed with the live CSFV solutions. The mixed samples were then subjected to Q-RT-PCR analysis to determine the titer of the live CSFV particles in the mixture of live and inactivated virus solutions.

2.7. Statistical analysis

All experiments were performed at least 3 times and the standard errors of means (SEM) of all data were calculated. The results