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Rapid Recovery of Classical Swine Fever Virus Directly from Cloned cDNA

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Abstract

The reverse genetics for classical swine fever virus (CSFV) is currently based on the transfection of in vitro transcribed RNA from a viral genomic cDNA clone, which is inefficient and time-consuming. This study was aimed to develop an improved method for rapid recovery of CSFV directly from cloned cDNA. Full-length genomic cDNA from the CSFV Shimen strain, which was flanked by a T7 promoter, the hepatitis delta virus ribozyme and T7 terminator sequences, was cloned into the low-copy vector pOK12, producing pOKShimen-RzT. Direct transfection of pOKShimen-RzT into PK/T7 cells, a PK-15-derived cell line stably expressing bacteriophage T7 RNA polymerase, allowed CSFV to be rescued rapidly and efficiently, i.e., at least 12 h faster and 31.6-fold greater viral titer when compared with the in vitro transcription-based rescue system. Furthermore, the progeny virus rescued from PK/T7 cells was indistinguishable, both in vitro and in vivo, from its parent virus and the virus rescued from classical reverse genetics. The reverse genetics based on intracellular transcription is efficient, convenient and cost-effective. The PK/T7 cell line can be used to rescue CSFV directly from cloned cDNA and it can also be used as an intracellular transcription and expression system for studying the structure and function of viral genes.

Key words: classical swine fever virus, reverse genetics, T7 RNA polymerase, stable cell line

INTRODUCTION

Reverse genetics of RNA viruses based on full-length infectious cDNA clones of viral genomes has emerged in the field of molecular virology to overcome the problem of in vitro viral RNA manipulation. Reverse genetics of RNA viruses rescues the viruses or their mutants from the cloned cDNA of viral RNA genomes in permissive cells or susceptible hosts. Generally, a full-length cDNA of viral genome is inserted into a plasmid, allowing infection using the cDNA clone itself or RNA transcribed from the cDNA in vitro. There are at least three methods for reverse genetic manipulation. The first is based on in vitro infectious RNA transcripts, the second is based on infectious RNA transcribed from cells, and the third is based on infectious cDNA clones (directly based on DNA). The second method uses recombinant viruses (e.g., vaccinia virus and fowlpox virus) (Baron and Barrett 1997; Evans et al. 2000) or stable cell lines (Radecke et al. 1995; Xiu et al. 2007; Freiberg et al. 2008) expressing RNA polymerases (RNAP) (e.g., T7 RNA polymerase) for the transcription of cloned cDNA in permissive cells. T7 RNAP is a DNA-dependent RNA polymerase derived from T7 bacteriophage that specifically recognizes T7 promoter sequence and has transcriptional activity in prokaryotic or eukaryotic cells. The T7 RNAP gene can be integrated into the genome of mammalian cells, where it is highly expressed and stable in many cell types (Elroy-Stein and Moss 1990). Thus, T7 RNAP is widely used in mo-
molecular cloning and reverse genetic manipulation (Kwon et al. 2006).

A reverse genetic manipulation platform for classical swine fever virus (CSFV) was established (Meyers et al. 1996; Moormann et al. 1996), but the infectivity of the genomic RNA transcribed in vitro from the full-length genomic cDNA of CSFV was relatively low (Moormann et al. 1996). To improve reverse genetics methods for CSFV, van Gennip et al. (1999) established a stable swine kidney cell line that expressed T7 RNAP (SK6.T7), which they used to develop a one-step method for the recovery of infectious CSFV from full-length genomic cDNA clones of CSFV C-strain based on SK6.T7 cells. This method improved recovery of infectious CSFV from full-length cDNAs with a 200-fold increase in viral titer when compared with in vitro transcribed RNA. However, linearization of the cDNA clone prior to transfection was necessary to achieve efficient virus rescue (van Gennip et al. 1999).

The PK-15 cell line is a well-established continuous cell line derived from porcine kidney and is one of several cell types permissive to CSFV (including most wild-type and several vaccine strains). PK-15 cells are also susceptible to some other swine viruses. Moreover, PK-15 cells were shown to be similar to porcine macrophages, target cells of CSFV, but different from SK-6 cells with respect to type I interferon responses upon stimulation with double-stranded RNA (Ruggli et al. 2003). Thus, PK-15 cells may be more suitable for study of CSFV replication. The present study was aimed to establish an improved methodology for the direct recovery of CSFV from cloned cDNA without prior linearization, using a PK-15-derived cell line that stably expressed the T7 RNAP (Huang et al. 2011).

RESULTS

Detection of the rescued viruses

Infectious vShimen-RzTΦ and vShimen were successfully rescued from pOKShimen-RzTΦ and pOKShimen, respectively. The supernatants of rescued viruses were collected and titrated. Compared to vShimen rescued by in vitro transcription, a 31.6-fold higher viral titer was achieved for vShimen-RzTΦ (5.62×10⁴ vs. 1.78×10³) rescued by intracellular transcription at 120 h post-transfection. Real-time PCR showed that the progeny viruses replicated in PK/T7 cells, and the total viral RNA and negative strand RNA of vShimen-RzTΦ were comparable with that of vShimen and wtShimen, respectively (Fig. 1).

PK-15 cells infected with vShimen-RzTΦ and vShimen were analyzed by IFA to test whether viral protein was expressed in the progeny viruses. PK-15 cells infected with wtShimen were used as the positive control and mock-infected PK-15 cells were also analyzed by IFA as a negative control. The results indicated that viral proteins were expressed in PK-15 cells infected with vShimen-RzTΦ or vShimen (Fig. 2).

Morphology of the rescued viruses

The morphology of the rescued viruses was investigated further by examining the PK-15 cells infected with vShimen-RzTΦ or vShimen using electron microscopy. As shown in Fig. 3, viral particles were spherical with a diameter of approximately 30-50 nm.

Genetic tags in the rescued viruses

The PCR products of vShimen-RzTΦ, vShimen and wtShimen were analyzed by digestion with Ava I to confirm the presence of genetic tags in rescued viruses. PCR products from samples of vShimen-RzTΦ and vShimen could be cleaved into two fragments using Ava I (Fig. 4).
Growth kinetics of the rescued viruses

The growth kinetics of vShimen-RzTΦ on PK-15 cells was compared with vShimen and wtShimen (Fig. 5). All viral titers reached their maximum value at 72 h post-infection (hpi). The titers of vShimen-RzTΦ and the paternal virus were $10^{5.5}$ and $10^{5.75}$, respectively. All viral titers began to decline at 96 hpi. The growth kinetics of the recovered viruses and the parent virus were similar.

Clinical features and pathological changes of experimentally infected pigs

All pigs in groups A, B and C showed fever, shivering and anorexia at 2 d post-inoculation (dpi), followed by loss of appetite, lethargy, stiffness of gait, reddening of the conjunctiva and loose stools or diarrhoea. Death occurred at 7-9 dpi in groups A, B and C. At 14 dpi, all pigs died from infection. In contrast, the control pigs in group D remained healthy throughout the trial.

Inoculated pigs in groups A, B and C showed lesions typical of CSF, such as petechiae in the kidney, haemorrhages in the lymph nodes and urinary bladder, necrosis in the tonsils and infarcts in the spleen. No significant differences were found between the three groups. As expected, the control pigs did not exhibit
any pathological changes.

DISCUSSION

To date, two types of system are used for rescuing CSFV from cloned cDNA, i.e., in vitro transcription system and intracellular transcription system based on endogenous T7 RNAP. Moormann et al. (1996) were the first to rescue CSFV using an in vitro transcription system. However, the virus yield from in vitro transcribed RNA is usually low in swine kidney cells, which is mainly due to the degradability and instability of RNA. The in vitro transcription-based rescue system is also complicated and expensive.

More efficient rescue of CSFV requires the development of a system based on endogenous T7 RNAP. PK-15 cells are also known to be suitable for the propagation of the CSFV Shimen strain, because of their high permission for highly virulent CSFV and many other viruses of porcine origin. We have developed a PK-15-derived cell line (PK/T7) to stably express T7 RNAP (Huang et al. 2011). This cell line can be used to rescue CSFV directly from cloned cDNA and it can also be used as an intracellular transcription and expression system for studying the structure and function of viral genes.

A major improvement with the present procedure is the introduction of the HDV Rz, which forms an accurate 3’-end on the transcribed RNA in PK/T7 cells (data not shown) thereby enabling direct transfection of circular full-length cDNA without prior linearization and resulting in rapid viral recovery. The infectious clone pPRKf1c17 described by van Gennip et al. (1999) contained several additional nucleotides in between the genomic cDNA and HDV Rz, which resulted in RNA with a non-viral sequence in the 3’-end thereby reducing the viral recovery efficiency. The current study introduced the HDV Rz sequence followed by the TF sequences immediately downstream of the genomic 3’-end in pOKShimen-RzTΦ. The resulting transcript contains precise 3’-end which allows to avoid the linearization step and increase the efficiency of virus recovery.

We compared the rescue efficiency of the two procedures: direct transfection of PK/T7 cells with pOKShimen-RzTΦ and transfection of PK-15 cells with RNA transcribed in vitro from pOKShimen. At 120 h post-infection, the titer of vShimen-RzTΦ was 31.6-times that of vShimen, showing that intracellular transcription was more efficient than in vitro transcription. Intracellular transcription also reduced the time and cost of rescuing viruses by avoiding 3’-end linearization, transcription and RNA purification, which are necessary processes in the in vitro transcription system. In vitro transcription took 12 h longer than direct transfection, including time for linearization and transcription.

Sequence analysis showed that four fragments (5’-
UTR, E2, NS5B and 3′-UTR) in the genomic cDNA reverse-transcribed from vShimen-RzTΦ were identical to those from the parent virus. The progeny virus was rescued efficiently in different passages of PK/T7 cells (5th, 10th, 15th, and 20th passages) transfected with the plasmid pOKShimen-RzTΦ (data not shown). Virus was not detected in the negative control (normal PK-15 cells) transfected with the plasmid pOKShimen-RzTΦ. These results demonstrate that PK/T7 cells stably expressed T7 RNAP and that the virus rescue method based on intracellular transcription was repeatable and efficient.

Products were generated by RT-PCR, but not by PCR when the AMV reverse transcriptase was omitted, indicating that the RT-PCR products were derived from the RNA of the rescued virus rather than a plasmid DNA residue in the transfection mixture. PCR products were digested by the introduced unique restriction enzyme Ava I (genetic tag), suggesting that the rescued virus was not a contaminant of the parent CSFV. Furthermore, the characteristics of vShimen-RzTΦ were indistinguishable from vShimen and the wild-type Shimen strain, as shown by real-time RT-PCR, IFA, one-step replication kinetics and experimental infection. The rescued virus was shown to be stable, because the genetic tag was still present after at least seven passages in PK-15 cells.

CONCLUSION

Taken together, this study developed and characterized a new stable cell line (PK/T7) for in vitro transcription, and designed and verified the rapid and efficient method for CSFV recovery from cloned cDNA.

MATERIALS AND METHODS

Cells, viruses and plasmids

PK-15 cells, a cell line permissive to CSFV, and PK/T7 cells, a PK-15-derived cell line stably expressing bacteriophage T7 RNA polymerase (Huang et al. 2011), were cultured in Dulbecco modified Eagle’s medium (DMEM) (Hyclone, USA) supplemented with 10% fetal bovine sera (FBS) at 37°C in a humidified 5% CO2 incubator. The PK/T7 cell line (see below) was grown in the same medium supplemented with 600 mg mL−1 G418 (Sigma, USA). The highly virulent CSFV Shimen strain was propagated on PK-15 cells in DMEM with 2% FBS.

Construction of the full-length CSFV cDNA clones

CSFV viral RNA was extracted from PK-15 cells infected with wild-type Shimen (wtShimen) using a QIAamp Viral RNA Extraction Kit (Qiagen, USA) and reverse transcribed into cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany). Six overlapping DNA fragments that covered the complete genome of the CSFV Shimen strain were successively cloned into the pOK12 vector to construct pOKShimen. A genetic tag was also introduced at nt 8215 of the genomic cDNA by site-directed mutagenesis. Two fragments were amplified using two pairs of primers (P6F11267-11293 and P3UTRRz-R; Rz3UTR-F and PT7tNotI-R) to flank the Shimen strain genome with the HDV ribozyme (Rz) sequence (Perrotta and Been 1991) followed by the T7 terminator (TΦ) sequence (Dunn and Studier 1983). The fusion fragment of HDV Rz and TΦ was amplified by overlapping PCR using the two fragments and primers P6F11267-11293 and PT7tNotI-R, before it was inserted into the 3′-end of the genomic cDNA clone. This yielded pOKShimen-RzTΦ, which could automatically terminate a single transcription cycle and effectively form an accurate 3′-end for CSFV RNA.

Rescue of viruses

The full-length genomic cDNA clone pOKShimen was linearized using Srf I (Stratagene, USA) and in vitro transcribed with T7 RNAP (Promega, USA). After removing the DNA template using DNase I (RNase free) (Promega), the RNA transcripts were mixed with 6 μL of Lipofectin reagent (Invitrogen, USA) before transfection on PK-15 monolayers in six-well plate cell for 6 h. Cells were maintained in DMEM containing 4% FBS for 72 h. Transfected cells were blind passaged three times and supplemented with some normal PK-15 cells each time to increase viral titer. The virus recovered from pOKShimen was named vShimen.

The purified plasmid pOKShimen-RzTΦ was extracted using Wizard Plus Minipreps System (Promega) and precipitated with 95% ethanol. PK/T7 cells were frozen to 80% confluency in a six-well tissue culture plate and transfected with the purified pOKShimen-RzTΦ using lipofectin. Plates were incubated for 6 h at 37°C in a humidified 5% CO2 incubator. Cells were then washed three times with DMEM and maintained in DMEM supplemented with 4% FBS for a further 72 h. The transfected PK/T7 cells were blind passaged three times, as described above. After freezing and thawing three times, the recovered virus vShimen-RzTΦ was harvested from the supernatant by centrifugation.
Identification of rescued CSFV

Viral genomic RNA was extracted from the rescued virus supernatant and reverse transcribed into cDNA as the template for RT-PCR. The controls were the supernatant from wtShimen and PK-15 cells, which were treated using the identical method. vShimen and vShimen-RzTΦ RNA lacking reverse transcriptase were detected simultaneously using RT-PCR to show that the positive signal was derived from reverse-transcribed RNA and not from residual plasmid DNA present in the transfection mixture.

The viral RNA replication level was measured quantitatively using real-time RT-PCR, as detailed previously (Zhao et al. 2008). Briefly, a random primer and CSFV-F were used to synthesize cDNAs from the total or negative strand viral RNA, respectively, while the primer pair CSFV-F/CSFV-R and the TaqMan probe FAM-CSFV-v111 were used in an assay to detect rescued virus samples.

The specificity of the recovered viruses was demonstrated by growing PK-15 cells to 70% confluence in a 96-well tissue culture plate and infecting them with vShimen-RzTΦ, vShimen or wtShimen. After incubating for 48 h at 37°C, the infected cells were washed with PBS and fixed with absolute ethanol for 20 min at -20°C. Fixed cells were incubated with CSFV-specific E2 monoclonal antibody (Peng et al. 2008) at 37°C for 2 h, washed five times, and incubated with FITC-labeled goat anti-mouse IgG (1:100 dilution with PBS) (Sigma) at 37°C for 1 h. Cells were washed eight times and examined by fluorescence microscopy.

Rescued viruses were examined by electron microscopy to determine the morphology of the rescued viruses. PK-15 cells were grown overnight to 70% confluence and infected with vShimen and vShimen-RzTΦ. Infected PK-15 cells were cultivated for 48 h before ultrathin sections were prepared and examined by electron microscopy.

The site-directed mutagenesis at nt 8215 of the genomic cDNA introduced a new AvaI restriction site with no amino acid change in the ORF, thereby generating a difference in genomes of the rescued virus and the wild-type CSFV. A fragment of 757 bp located between nt 7744 and 8850 of the genomic cDNA was amplified from reverse transcribed cDNA using primers PTag-F and PTag-R (Table). The genetic tag in the 757-bp fragment was confirmed by digestion with AvaI.

Table Oligonucleotide primers used in PCR

| Primers | Sequences (5´→3´) |
|---------|-------------------|
| P1F-SalIT7-1-16 | GTCGAC TAATACGACCTCTATAGTAGTATACGAGGTTAGTT (introduced restriction site is underlined; T7 promoter is shaded) |
| P1R2681-2699 | TCCCCGTAACAGGACCTC |
| P2F2354-2375 | GTCGAC GACCATCTATCTGCTTGATAG |
| P2R2428-4257 | AGAATGTCTGCACCATGTC |
| P3F4073-4096 | CTTAAGGGAATAGTTGAGGGATATG |
| P3R6498-6521 | GGAGAGTAGAAACACGTCATGAG |
| P4F6302-6326 | GCTGGACTGAAATAGGGAATG |
| P4R8770-8793 | CCCCCTTGAGTTTTGACCATG |
| P5F8596-8619 | CTTGCGGCTACAGATGGAAGGAGC |
| P5R11627-11650 | ATGCGAATCCGTTCTGATCATG |
| P6F11267-11293 | CTGATCACAGAAGAAGACGCTGGTGAG |
| P6R12280-12298 | GCCGCCGCCGGCCTGATAGAAATTACCT | (introduced sites are underlined) |
| PmAvaI-F | TTAGTCAAGGAGAGTAAGCAC |
| PmAvaI-R | GCCATTATTATTCTCCTGAGATCT |
| P3UTRRz-R | TGGAGATGACATGGGCCGCCGCCCTGATTGAAATTACCT |
| PRz3UTR-F | AGGTAAATTTCCTAACGGCCCGGGTCGGCAGCTC |
| PT7tNotI-R | GCCGCCGCAATTCCTCCCCAGCCA |
| PTag-F | AGACCTAGTGCTCCAGAGA |
| PTag-R | CTGATGCTCATCCCTCAC |

Replication kinetics of the rescued CSFV

One-step growth curve was used to analyze the replication kinetics of the rescued viruses and wtShimen. Confluent PK-15 cells in a 12-well plate (approximately 10⁵ cells per well) were infected with vShimen-RzTΦ, vShimen or wtShimen at a multiplicity of infection (MOI) of 1. Infected cell cultures were harvested at 12-h intervals and the titer of infectious progeny virus was determined as median tissue culture infective dose (TCID₅₀) per mL using the Reed-Münch method (Reed and Münch 1938).

Experimental infection of pigs

Twelve 6-wk-old healthy pigs negative for CSFV RNA and anti-CSFV antibodies were randomly divided into three groups and housed separately in the animal facility at Harbin Veterinary Research Institute, China, under standard con-
conditions prescribed by the Institutional Guidelines. Pigs in groups A, B and C were inoculated intramuscularly (i.m.) with 10^5 TCID_50 vShimen-RzTΦ, vShimen or wtShimen, respectively. Group D of three pigs were mock inoculated i.m. with DMEM. Following viral inoculation, rectal temperature and clinical signs were recorded on a daily basis. The tonsils, spleen, kidney, liver, lung, urinary bladder, and lymph nodes were collected from the pigs after death from infection or after euthanasia at the end of the trial for evaluation of pathological changes. The study protocol was approved by the Institutional Animal Care and Use Committee.

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