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Construction of a Chimeric Virus Expressing Mutation Sequences of Classical Swine Fever Virus Yunnan Strain

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Abstract: Infectious cDNA clones are a prerequisite for directed genetic manipulation of RNA viruses. To explore the role of mutations in Classical Swine Fever Virus (CSFV) Yunnan strain which caused the atypical clinical signs in pigs, a new pSM derived from CSFV Shiman strain has been constructed and subsequently replaced by the mutation sequences of CSFV YN strain isolated by the laboratory at the positions 1510-1532, 2471-2658, 3152-3176 and 11785-11816 using the targeted recombination strategy to enable rescue of chimeric CSFV. The results showed that chimeric CSFV (vSM-YN) was successfully rescued from PK-15 cells by transfection of the chimeric CSFV RNA transcripts and identified by whole genome sequence analysis, immunofluorescence antibody assay and ELISA detection. Sequencing of the pAC-SM-YN revealed a high genetic stability and the complete genome sequences of rescued viruses vSM-YN after extensive passages in PK-15 cells showed that modifications in pSM were stably maintained. The results indicate that targeted recombination-mediated mutagenesis provides a powerful tool for expediting the construction of novel RNA genomes and should facilitate further study of the pathogenic mechanism of CSFV leading to atypical CSF.

Key words: RNA, CSFV, chimeric virus, construction, genetic stability, infectious molecular clone

INTRODUCTION

Classical Swine Fever (CSF) caused by Classical Swine Fever Virus (CSFV) is a highly contagious disease of pigs and wild boars. CSFV together with Border Disease Virus (BDV) and Bovine Viral Diarrhea Virus (BVDV) belongs to the genus Pestivirus within the family Flaviviridae. All pestiviruses are structurally and genetically closely related (Gallei *et al*., 2005). BVDV and BDV are able to infect pigs and ruminants whereas CSFV only infects pigs under natural conditions (Liess and Moennig, 1990). The pestiviruses have single stranded positive sense RNA genome of about 12.3 kb, encoding a single Open Reading Frame (ORF). This ORF is translated into a polyprotein that is processed into four structural proteins (nucleocapsid protein C, envelope glycoproteins E^{rns}, E1 and E2) and eight nonstructural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) by viral and host-cell proteases (Meyers and Thiel, 1996). To present, outbreaks of CSF in domestic pigs and wild boards still occur in many countries causing significant economic losses (Artois *et al*., 2002; Edwards, 2000).

Rapid response and control of emerging CSFV require high-throughput strategies for the manipulation and recovery of recombinant viruses by reverse genetic strategies. Reverse genetics has the ability to introduce a mutation by design and is currently available in *Escherichia coli*, yeast and the mouse (Soriano *et al*., 1995). Through deletions, point mutations, amplifications, insertions, inversions and translocations of the target genes, the DNA sequence was altered (Boyer and Haenni, 1994). Then by *in vitro* transcription and sensitive cell transfection, the progeny of virus was obtain for further investigating the regulation and expression of the viral genome and the molecular pathogenesis of the virus (Justice *et al*., 2011). It has been shown that genome segments from different pestiviruses can be combined, leading to recombinant viruses. These chimeric CSFVs represent a powerful approach to combining the high-protection potential of conventional live-attenuated vaccines with the DIVA (Differentiating Infected from Vaccinated Animals) principle. Several chimeric viruses have been developed over the years. In particular, chimeric pestiviruses with substitution of the entire the *E2* or E^{rns} gene by the respective gene of a heterologous

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virus strain or another pestivirus have been described (De Smit *et al*., 2001; Li *et al*., 2009; Sun *et al*., 2008; Widjojoatmodjo *et al*., 2000).

In this study, researchers described the construction and assembly of a full-length molecular clone for chimeric vSM-YN expressing the genes that were replaced with the respective mutated sequences 1 510-1532, 2471-2658, 3152-3176 and 11785-11816 from CSFV YN strain. Recombinant viruses were also isolated and expressed in PK-15 cells. It indicated that the availability of genetically defined and stable CSFVs facilitated the functional study of viral proteins or RNA structures and also the development of new marker vaccine candidates.

MATERIALS AND METHODS

Viruses and cells: High virulent CSFV Shimen strain was isolated from pigs and CSFV Yunnan strain named CSFV-YN-2009 was also isolated from pigs occurring atypical CSF symptoms. Then, the viruses were preserved in the laboratory (Laboratory of Viruses, College of Animal Science and Technology, Yunnan Agricultural University). The plasmids pYN containing full length cDNAs of CSFV Yunan strain and pSM containing full length cDNAs of CSFV Shimen strain were also constructed and preserved in the laboratory. PK-15 cells were provided by China Veterinary Culture Collection Center.

Construction of chimeric pSM-YN: Recombinant plasmid pSM containing the full length gene of CSFV Shimen strain used as templates for long PCR or for screening by restriction enzyme digestion was purified from 4 mL overnight cultures of *Escherichia coli* TOP10 (TIANGEN) and amplified by PCR and got six fragments. Furthermore, pYN containing the full length gene of CSFV Yunnan strain was amplified and got a fragment at position of 2471-2658. Then, seven fragments were cloned into pMD18-T (Takara). Positive vectors were screened and sequenced by Shanghai biological Engineering Co. and the right clones named P1, P2, P3, P4, P5, P6 and P7 were picked. Using SOE-PCR Method, P1, P2, P3, P4 and P5 fragments were constructed to the 5' half-length cDNA. P6 and P7 were connected to the 3' half-length cDNA by overlap extension PCR. Finally, the 5' half-length cDNA and the 3' half-length cDNA were co-transformed into *Escherichia coli* with pACYC184 vector (Invitrogen) by electroporation with a Bio-Rad Gene Pulser at 1800 V, 25 μF and 200Ω. The transformed bacteria were inoculated onto LB agar plates containing 50 μg mL^{-1} kanamycin. The recombinant plasmid was identified by PCR, PacI digestion and sequencing. The resulting recombinant named pAC-SM-YN was identified (Fig. 1).

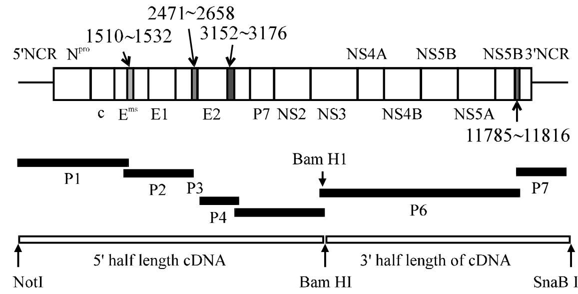

Fig. 1: The construction schematic of chimeric CSFV genome. Schematic representation of the CSFV genome organization and chimeric virus constructed in this study. Insertion of mutation sequences in place of the CSFV Shimen strain and the digestion sites are shown

Full-length RNA transcription: Using Plasmid Midipure kit (Qiagen), a large amount of pAC-SM-YN was extracted. The 2 mg of the respective cDNA construct was linearized with Psy I restriction enzyme and purified by phenol extraction and ethanol precipitation. Transcription was carried out *in vitro* using MEGAscript® T7 kit (Ambion) according to the instructions. Briefly, the transcription mixure containing 40 mM Tris-HCl (pH 7.5); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 0.5 mM (each) ATP, CTP, GTP and UTP; 100 mg mL^{-1} bovine serum albumin, 50 U T7 RNA polymerase and 15 U of RNA guard was incubated at 37°C for 1 h then the reaction mixture was passed through a spun column and further purified by phenol extraction and ethanol precipitation.

Rescue of chimeric virus: To produce the recombinant virus, 85% confluent PK-15 cells in a 6-well plate were transfected with 5 μg of pAC-SM-YN linearized with PacI enzyme using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, CA, USA) according to the manufacturer's recommendations. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) pestivirus-free Fetal Calf Serum (FCS), 100 U of penicillin/mL and 100 U of streptomycin/ml at 37°C in a humidified 5% CO_2 incubator. The 96 h post-transfection, cells were split and seeded as appropriate for subsequent analyses.

RT-PCR: The pAC-SM-YN linearized with PacI enzyme was transfected to PK-15 cells in 6 well cell culture plates and sub-cultured for 5 times. Then, total RNA in cell cultures was isolated using TRIZOL reagent following the manufacturer's instructions (Invitrogen) and CSFV

Table 1: Primers used for RT-PCR to synthesize seven cDNA clones for creating pSM-YN

Names	Nucleotide sequence 5'→3'	Size (bp)
P1	AATGCGGCCGCTATACGAGGTTAGCTCGGTCC	1532
	ATATTTCTGTCGTACCTGCAAGGCACGGCGCACTCCTTATTTGG	
P2	CCTTGCAGGTACGACAGAAATATCGATGTTAACGTGGTCACC	939
	GTACCTGTAATCTTCCTTGC	
P3	CTATCTCATCGACCGATGAG3	187
	GCCCTCTTGTGTAATGATG	
P4	CCTGGCATCATTACACAAGAGGGCCTTTACTCACTTCCGTGACATTC	518
	ACGCCATCTCTGTTGCAGTCTGTAAATCTACTATTCTGTAACCTG	
P5	CACAGACTGCAACAGAGATGGCGTTTGTAATCAGCGCAGAGGGGAG	3260
	ATGGATCCTCTCCACTGTAATAG	
P6	GAGGATCCATCTAACCTGAGGGTG	5349
	AAGTCTTGTAGTAATCTTCTGCTGCTATGCGTTCCAGGCCCCGAGTACAG	
P7	ACGCATAGCAGCAGAAGATTACTACAAGACTTTGTCAATATGGGTGTTA	513
	GTATACGTAGGGCCGTTAGGAAATTACCTTAG	

specific gene fragments and DNA sequencing were determined by Reverse Transcription Polymerase Chain Reaction (RT-PCR). RT-PCR was performed using the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The PCR reaction was carried out at 1 cycle of 50°C for 3 min and 95°C for 10 min followed by 40 cycles of 95°C for 30 sec, 55°C for 45 sec and 72°C for 30 sec. Researchers used 18SrRNA expression as an internal control. Quantification of gene expression was performed by the $2^{-\Delta\Delta C_T}$ Method. Specific primer sequences were designed using Oligo 6.0 Software and synthesized in BIOSUNE Biological Technology Corp (Shanghai, China) and the sequences of the primers were shown in Table 1.

Indirect Immunofluorescent Assay (IFA): The vSM-YN was added to PK-15 cells in 6 well cell culture plates. After 5th passages, antibodies were determined by CSFV indirect fluorescent antibody kits. The fluorescence signal was detected with an inverted fluorescence microscope (Nikon, Japan). Anti-CSFV fluorescence antibody kits were purchased from China Institute of Veterinary Drugs Control.

Enzyme-Linked Immunosorbent Assay (ELISA): The vSM-YN was used to infect PK-15 cells and repeated subcultures were taken. After 5, 10 and 15 passages in PK-15 cells, cell cultures were frozen and thawed for 3 times and centrifuged at 8000 r min^{-1} for 10 min then supernatants were collected to test for the presence of CSFV-specific Antibodies (Abs) using the CSFV Antibody Test kits according to the manufacturer's instructions. Briefly, 96-microtiter well plates were coated with the samples (50 μL well^{-1}) and incubated 2 h at 25°C and then washed 3 times with PBS containing 0.05% Tween-20 (PBST) and blocked with 0.5% nonfat milk in PBS for 1 h at 25°C. The plates were washed and treated with peroxidase-conjugated goat anti-mouse IgG for 1 h at 25°C. After washing with PBS, peroxidase substrate 3,3', 5,5'-Tetramethylbenzidine (TMB) was added and this was followed by incubation for 10 min at 25°C. The reaction was finally stopped by adding 0.5 M H_2SO_4. The Optical Density (OD) value of each well was read at 450 nm on an ELISA reader (Bio-Rad, Hercules, CA, USA). CSFV Antigen test kits were purchased from Beijing IDEXX Yuanheng Biotechnology Co., Ltd.

RESULTS AND DISCUSSION

Generation of a CSFV full-length cDNA: Using pSM containing *CSFV* gene of Shimen strain as a template and primers based on the mutation sites of CSFV Yunnan strain, six fragments named P1, P2, P4, P5, P6 and P7 with mutation sites of CSFV Yunnan strain were amplified by PCR. Additionally, using p-YN containing CSFV Yunnan strain full-length gene as a template, a fragment named P3 was amplified and the expected results were got (Fig. 2). The results of sequencing proved that mutation sequences 1510-1532, 2471-2658, 3152-3176 and 11785-11816 of atypical CSFV Yunnan strain were successfully inserted into the corresponding gene regions of CSFV Shimen strain.

Identification of recombinant pAC-SM-YN: Using overlap extension PCR Method, seven cDNA clones were co-transformed into *Escherichia coli* with pACYC184 vector and got recombinant pAC-SM-YN. Using BamH I restriction enzyme, pAC-SM-YN was digested and yielded a band of 16.5 kb in size. In addition, digestion with Not I and SnaB I enzymes, a band with a size of 12298 bp and a band with a size of 4245 bp were produced which were consistent with the expected results (Fig. 3). Furthermore, the enzyme digested pAC-SM-YN was sequenced, the result indicated that infectious recombinant plasmids containing the full length CSFV cDNAs with mutation genes of Yunan virus strain were successfully constructed.

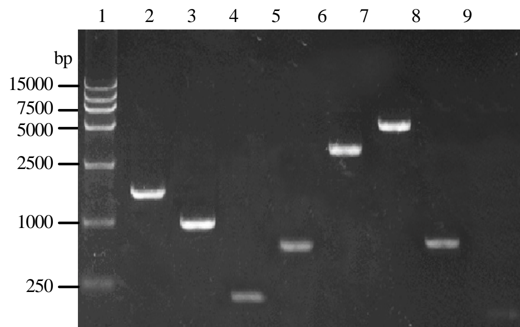

Fig. 2: Amplification of chimeric CSFV genomic fragments by PCR. Six fragments named P1, P2, P4, P5, P6 and P7 with mutation sites of CSFV Yunnan strain were amplified by PCR using pSM containing *CSFV* gene of Shimen strain as a template. A fragment named P3 was amplified using pYN containing CSFV Yunnan strain full-length gene as a template. 1: DL15 000 Marker; 2-8. The PCR products of Chimeric CSFV genome P1-P7 fragments; 9: Negative control

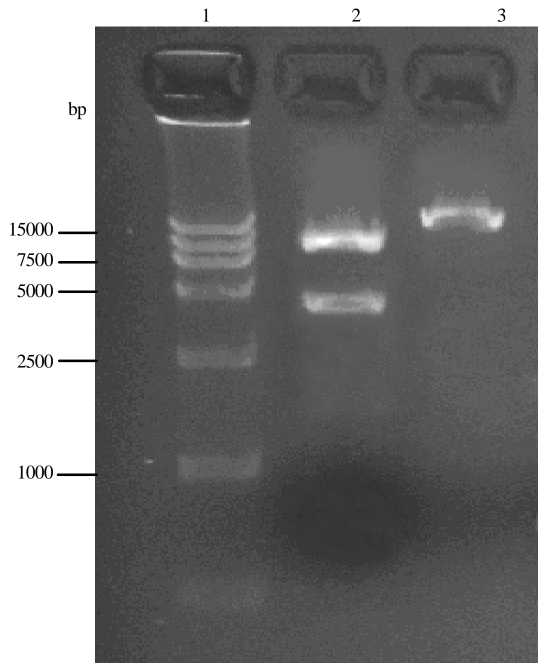

Fig. 3: Identification of the chimeric CSFV infectious clone pAC-SM-YN by restriction enzyme digestions. Digestion with Not I and SnaB I enzymes, pAC-SM-YN was digested and a band with a size of 12298 bp and a band with a size of 4245 bp were produced. A band of 16.5 kb in size was yielded using BamH I restriction enzyme. 1: DL15 000 Marker; 2: Digestion by Not I and SnaB I; 3: Digestion by BamH I

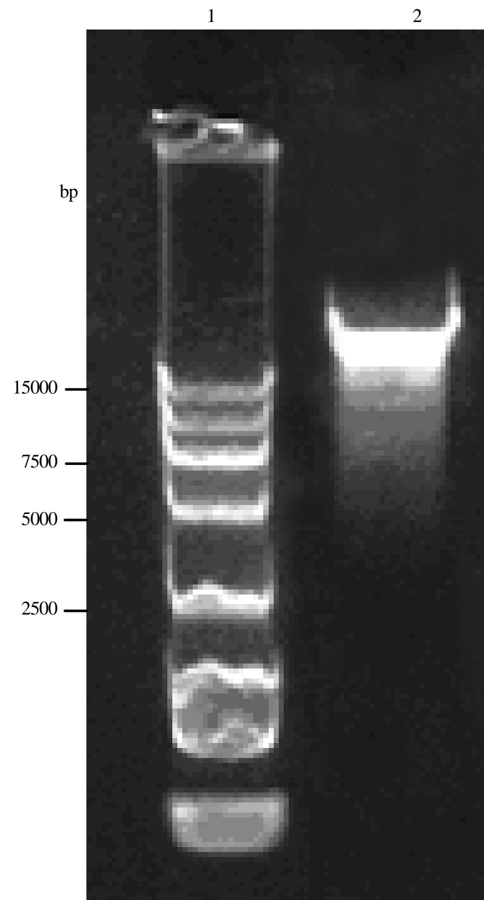

Fig. 4: The transcription result of chimeric CSFV full-length cDNA *in vitro*. pAC-SM-YN was linearized with Psy I restriction enzyme and purified by phenol extraction and ethanol precipitation. Transcription was carried out *in vitro* using MEGAscript® T7 Kit. 1: DL15 000 Marker; 2: Transcripts of Chimeric viruses *in vitro*

Constructed pAC-SM-YN was then linearized by Psy I restriction enzyme and purified by phenol extraction and ethanol precipitation. Transcription was carried out *in vitro* using MEGAscript® T7 kit and RNA transcript product was identified in Fig. 4.

Chemeric virus growth in PK-15 cells: To rescue the recombinant virus, the pAC-SM-YN linearized with PacI enzyme was transfected to PK-15 cells. The IFA showed that the PK-15 cells the presence of full-length SM-YN transcripts exhibited a strong green fluorescence after 5 passages (Fig. 5), indicating that chemeric virus named vSM-YN was rescued from PK-15 cells.

To further confirm robust vSM-YN replication, researchers performed RT-PCR and clearly demonstrated the presence of 600 bp full-length vSM-YN transcripts

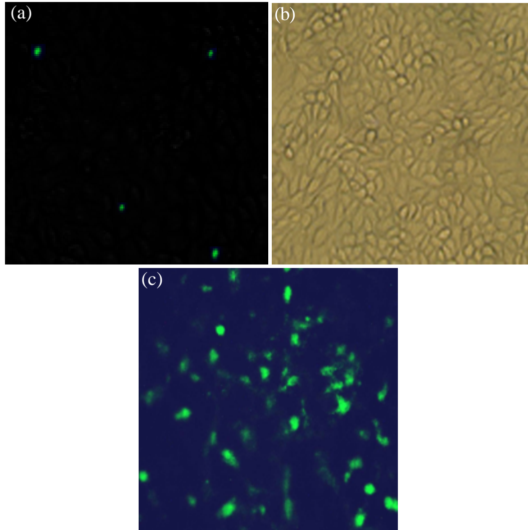

Fig. 5: Detection of CSFV in PK-15 cells infected with the recombinant vSM-YN by indirect Immunofluorescent Assay (IFA). After 5th passages, the fluorescence signal in PK-15 cells infected vSM-YN was detected with an inverted fluorescence microscope; a) and b) Negative control; c) PK-15 Cells infected with vSM-YN

during virus infection (Fig. 6). An indirect ELISA was used to monitor the CSFV-specific antibody levels. Consonant with these data, all samples collected from cell cultures without vSM-YN infection were negative for CFSV antibody, however cells infected with vSM-YN were positive for CFSV antibody and developed significant antibody responses after 2 passages in PK-15 cells (Fig. 7). A further increase in antibody level was observed after 10 passages. The results earlier demonstrated that vSM-YN rescued from PK-15 cells had the properties of CSFV.

Classical Swine Fever (CSF) caused by CSFV is a highly contagious disease of pigs and wild boars leading to big economic losses in many countries. So far, the epidemic potential of CSFV is still high in developing countries therefore, life cycle, biology, gene function and pathogenic mechanisms of CSFV clearly warrant additional studies. Developing full-length infectious cDNAs of emerging RNA viruses expressing other genes is usually used to develop the new kinds of vaccines. In this instance, researchers synthesized a molecular clone from the full-length genomes of CSFV Shimen strain expressing mutated sequences of CSFV Yunan strain and isolated recombinant viruses in PK-15 cultures.

The previous findings have suggested that a full-length cDNA clone that rescues infectious viral progeny would be an excellent tool for the functional characterization of viral gene products, analysis of virus and RNA replication, determination of virulence factors and elucidation of mechanisms involved in viral pathogenesis. Such a clone has served as an invaluable tool for developing a CSF marker vaccine. Additionally, the generation and use of infectious CSFV clones have provided new opportunities for understanding and characterizing the mechanisms of viral replication and

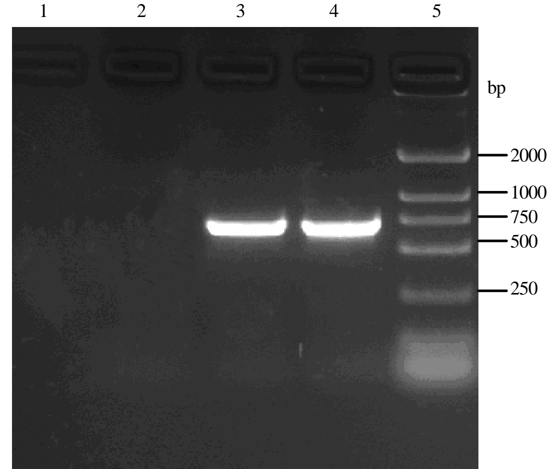

Fig. 6: The detection of the rescued chimeric CSFV by RT-PCR. The pAC-SM-YN linearized with PacI enzyme was transfected to PK-15 cells and sub-cultured for 5 times. Then, total RNA in cell cultures was isolated and CSFV specific gene fragments and DNA sequencing were determined by RT-PCR. 1: Untransfected PK-15 cells; 2: Only liposome transfected PK-15 cells; 3: Chimeric virus-infected PK-15 cells; 4: Positive control; 5: DL2 000 Marker

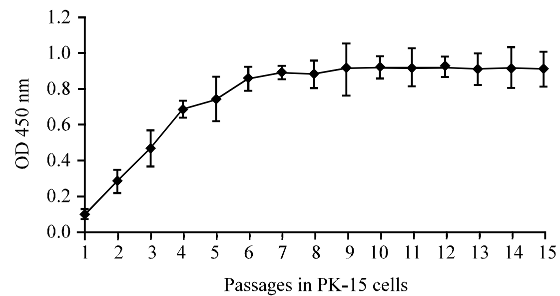

Fig. 7: Detection of antibody induced by vSM-YN in PK-15 cells using indirect ELISA. The ELISA was carried out as described by the manufacturer's instruction. Data were shown as mean±standard error of the mean

pathogenesis (Risatti *et al*., 2005). To date, reverse genetics has enabled the identification of CSFV proteins or protein domains that determine virulence and host range as well as facilitated the rational design and development of new vaccines against CSF. The studies also suggest that all infectious cDNA clones that are routinely used for molecular studies have been established from the moderately virulent Alfort/187 strain of CSFV (Ruggli *et al*., 1996), the attenuated C-strain (Moormann *et al*., 1996), the highly virulent Eystrup strain and the avirulent Riems (Mayer *et al*., 2003). Most of the novel modified CSF vaccines including deletion mutants, chimeric viruses and replicons have been developed by constructing cDNA clones of CSFV and BVDV (Meyers *et al*., 1996; Vassilev *et al*., 1997). These have the potential for inducing immunity to an extent similar to that of conventional live attenuated vaccines and present the possibility of discriminating vaccinated from infected animals. Several live chimeric pestiviruses have been constructed where the glycoproteins E2 and Erns were exchanged either partially or entirely between BVDV and CSFV (Reimann *et al*., 2004; Sun *et al*., 2013; Van Gennip *et al*., 2002) or between BVDV and BDV (Rasmussen *et al*., 2007). Pigs vaccinated with these chimeras were protected from challenge infection to a varying degree. In this study, researchers also successfully constructed a chimeric virus expressing the mutation sequences which are involved in the virulence of CSFV Yunan strain leading to atypical CSF symptoms. To build a stable chimeric virus, SOE-PCR Method was used to insert the mutation sequences of Yunnan strain into the genome CSFV Shimen strain without changing the bases or restriction sites in the viral genome of the chimeric virus. Furthermore in the process of constructing the chimeric virus genome, high-fidelity long PCR was used to clone the whole-genome. Low-copy number (15 copy number) vector pAC was used to construct chimeric infectious clones of CSFV to avoid the mutation in using high copy number plasmid vector pUC, pEMBL and pBluescript (Hofmann *et al*., 2000; Hurrelbrink *et al*., 1999). Therefore, the mutations of clones may occur were avoided in the present study, researchers successfully construct a stable pAC-SM-YN.

Cells such as BHK-21, PK-15 and SK6 cells are usually used to transfect or package the viruses. It found that these cells had different efficiency on the transfecting or packaging of the virus. Although, the transfection efficiency of BHK-21 cells is higher than PK-15, electrically transfecting RNA of C strain to BHK-21 cells has shown a small number of positive cells (Meyers *et al*., 1967). Furthermore, BHK-21 cell is not the host cell of CSFV. So, researchers did not choose BHK-21 cell as transfected cells. An investigation demonstrated that CSFV Shimen strain was suited for SK6 cells (Porntrakulpipat *et al*., 2010). In this study, researchers selected PK-15 cells as transfected cells. After continuous passages, direct fluorescence detection showed that the number of chimeric vSM-YN infected positive cells was increased in the fourth generation. ELISA test also confirmed that after 5 passages, the results were positive and the differences were not significant, indicating that the chimeric virus constructed successfully and stably grew in PK-15 cells.

CONCLUSION

In summary, the present study shows that targeted recombination-mediated mutagenesis of pAC-SM-YN containing the full length CSFV cDNA is successfully constructed and vSM-YN is rescued from PK-15 cells. The system shows high genetic stability of the pAC-SM-YN constructs within *E. coli* and the chimeric viruses rescued can be efficiently and stably propagated in PK-15 cells and this represents a suitable system for the production of virus stocks for future marked vaccine experiments or for exploring molecular mechanism of pathogenesis and immunity of CSFV. The strategies employed in this study have applicability not only for CSFV but should be adaptable to the study of other RNA viruses.

ACKNOWLEDGEMENT

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