

Establishment and characterization of a chimeric infectious cDNA clone of classical swine fever virus

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Summary. – Classical swine fever virus (CSFV) causes a highly contagious disease among swine that has an important economic impact worldwide. There are two important CSFV strains in China, Shimen and hog cholera lapinized virus (HCLV). Shimen strain is highly virulent while HCLV, also referred to as C-strain, is a live attenuated vaccine strain considered to be one of the most effective and safest live vaccines. In this study, a chimeric infectious cDNA clone of CSFV named pT7SM-c was engineered by replacing the E^{ms} genomic region of an infectious clone of CSFV Shimen strain, pT7SM, with the same region obtained from HCLV. RNA transcripts of pT7SM-c containing an engineered *EcoRI* site that served as a genetic marker were directly infectious in PK15 cells. The rescued virus vT7SM-c showed similar growth kinetics and cytopathic effect with the parental virus vT7SM in the cells. The chimeric infectious cDNA clone can be used as a practical tool for further studying of the virulence, protein function and pathogenesis of CSFV through genetic manipulation.

Keywords: classical swine fever virus; chimeric infectious cDNA clone; E^{ms}

Introduction

Classical swine fever virus (CSFV) is the etiological agent of classical swine fever (CSF), also known as hog cholera, a devastating disease of swine and wild boar, which causes significant economic losses in the pig industry worldwide. CSFV, together with bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) constitutes the genus *Pestivirus* within the family *Flaviviridae* (Thiel *et al.*, 1991; Becher and Thiel, 2002). Like other members of the genus *Pestivirus*, CSFV is a small enveloped virus with a positive-sense single-stranded RNA genome of about 12.3 kb in length, which contains 5' untranslated region (UTR), 3' UTR and one open reading frame (ORF) coding for a polyprotein of about 3898 amino acids. The viral polyprotein is co- and post-transla-

tionally processed by both viral and host proteases into 12 mature proteins including N^{pro}, C, E^{ms}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Rumenapf *et al.*, 1993).

The effective prevention and control of CSF usually depends on HCLV vaccine in China and many other countries of the world. The HCLV strain is a live attenuated vaccine strain that was obtained from the highly virulent strain of CSFV by 480 passages in rabbits in China during the 1950s. However, the mechanism of attenuation of this successful vaccine strain is still unknown. Recently, studies of determinants of virulence and attenuation mechanism of CSFV progress rapidly, which is attributed to the successful construction of infectious cDNA clones of CSFV (Meyers *et al.*, 1996; Ruggli *et al.*, 1996; de Smit *et al.*, 2000; Wu *et al.*, 2003; Park *et al.*, 2012). As reported, the deletion of the viral gene N^{pro} would lead to CSFV attenuation (Mayer *et al.*, 2004), E2 glycoprotein of CSFV was a virulence determinant, while E1 glycoprotein of CSFV would affect viral virulence in swine (Risatti *et al.*, 2005a,b), and the introduction of a continuous 12-nt (CUUUUUUCUUUU) insertion in viral 3' UTR might be a novel attenuation mechanism of CSFV (Wang *et al.*, 2008).

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Abbreviations: CSFV = classical swine fever virus; HCLV = hog cholera lapinized virus; PK15 = porcine kidney 15 cells

E^{rms} , a structural protein of CSFV with RNase activity (Hulst *et al.*, 1998), was found to induce apoptosis in lymphocytes of several species (Bruschke *et al.*, 1997). Since pestivirus infection is characterized by leukocyte reduction and immunosuppression, E^{rms} is considered to be associated with the pathogenesis of CSFV. Further studies indicated that inactivation of the RNase activity of CSFV E^{rms} resulted in cytopathic effect and virus attenuation in swine (Hulst *et al.*, 1998; Meyers *et al.*, 1999). However, E^{rms} shows RNase activity in both virulent and avirulent CSFV strains. Obviously, RNase activity of E^{rms} is not a good explanation to HCLV attenuation. In order to investigate the role of E^{rms} in CSFV attenuation, a chimeric infectious cDNA clone was constructed that contains all genetic regions of Shimen strain except that of E^{rms} from HCLV strain. This chimeric infectious cDNA clone can be used as a practical tool for further studying the virulence, protein function and pathogenesis of CSFV.

Materials and Methods

Viruses and cells. The porcine kidney 15 (PK15) cell line was obtained from China Center for Type Culture Collection. PK15 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were maintained at 37°C in a humidified atmosphere containing 5% of CO₂. The CSFV highly virulent strain Shimen and vaccine strain HCLV were provided by China Institute of Veterinary Drug Control.

Construction of chimeric cDNA clone. An infectious cDNA clone of CSFV strain Shimen named pT7SM was constructed previously (Wu *et al.*, 2003). Seven cDNAs amplified by reverse-transcriptase PCR (RT-PCR) were cloned into the low-copy-number plasmid pBR322 to obtain pT7SM. Gene E^{rms} is located in cDNA2.

Utilizing the swinging of the third base in a code, the T at site 1219 was replaced with A to create a unique *EcoRI* restriction site (GGAATTCAG) in E^{rms} genetic region of cDNA2 with the primer pairs of 5'-CCACATGATAGGGGGAGAGG-3' and 5'-CGTTACCTTAAGTCGTACGAT-3'; 5'-CGGAATTCAGCATGCTATGTACC-3' and 5'-GGGGCACCAAACCAGGTTTTGCTT-3'. Gene E^{rms} from the HCLV strain of CSFV was amplified using RT-PCR with a primer pair of 5'-CGGAATTCAGCATGCTATGTACC-3' and 5'-GGGGCACCAAACCAGGTTTTGCTT-3'. The genomic region of E^{rms} of plasmid pT7SM was replaced with the amplified E^{rms} from HCLV strain to generate a chimeric clone pT7SM-c by using restriction enzymes *EcoRI* and *BshN1* (NEB, USA) (Fig. 1a).

Rescue of viruses. Full-length genomic clone pT7SM and pT7SM-c were linearized with *SacII* and the 3' UTR end of *SacII* site was blunted by T4 DNA polymerase (NEB, USA). Then the linearized clones were *in vitro* transcribed by the T7 RNA polymerase (Promega, USA). After removing the DNA template by DNase I (RNase free) (Promega, USA), the RNA transcripts were mixed with 10 µl Lipofectin2000 (Invitrogen) for transfection on PK15 monolayer in 6-well cell plate for 6 hr. Then the cells were maintained in Dulbecco's Modified Eagle Medium (4% fetal calf serum) for 48 hr. The viruses recovered from full-length clones were named vT7SM and vT7SM-c, respectively.

The transfected cell cultures were frozen and thawed for three times for re-infection on PK15 cells to check the infectivity of clones. After five passages, the infection in cell culture was detected by RT-PCR. Furthermore, indirect immunofluorescence assay was performed to make sure that vT7SM and vT7SM-c were rescued in the cells by using the CSFV-specific rabbit anti- E^{rms} serum (Chen *et al.*, 2007) as primary antibody and mouse anti-Rabbit Rhodamine-conjugated IgG as secondary antibody (Beijing Zsbio).

Growth curves of Shimen, vT7SM and vT7SM-c on PK15 cells. Growth kinetics of the chimeric virus and parental strains was characterized by infection of PK15 cells seeded in a 6 well plate (MOI = 0.1). Culture supernatant was collected at 12, 24, 36, 48,

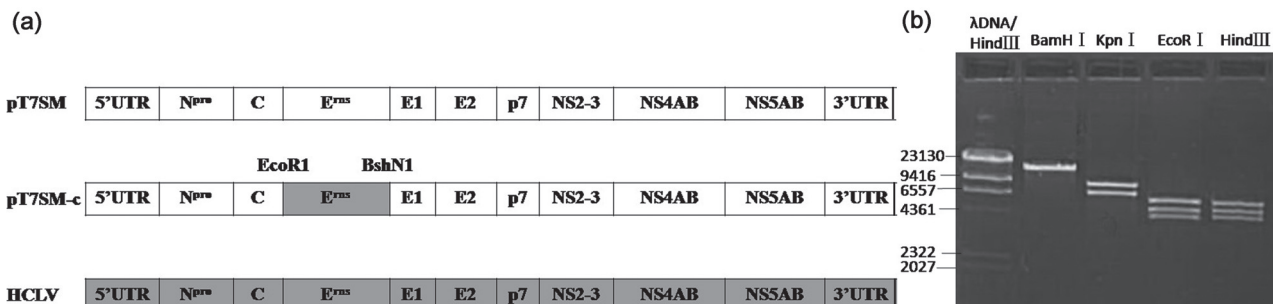


Fig. 1

Construction and identification of chimeric full-length cDNA clone pT7SM-c

(a) A schematic genome figure of chimeric and parental strains and construction of chimeric cDNA clone. The genomic regions of parental viruses, pT7SM (blank) and HCLV (gray), and the locations of the restriction enzyme sites used for genetic manipulation are indicated. (b) Identification of pT7SM-c by enzyme digestion.

Table 1. Distribution of enzyme restriction sites in pT7SM-c

Restriction enzyme	Restriction site (nt)	Band size after digestion (bp)
<i>Bam</i> HI	6436	16038
<i>Kpn</i> I	4448, 11522	7074+8964
<i>Eco</i> RI	1217*, 5562, 5799, 1299	6591+4956+4345+237 (not seen)
<i>Hind</i> III	752, 1255, 5711, 11728	6017+5062+4456+503 (not seen)

*Eco*RI restriction site at 1217* was introduced into E^{ms} to facilitate the genetic segment exchange.

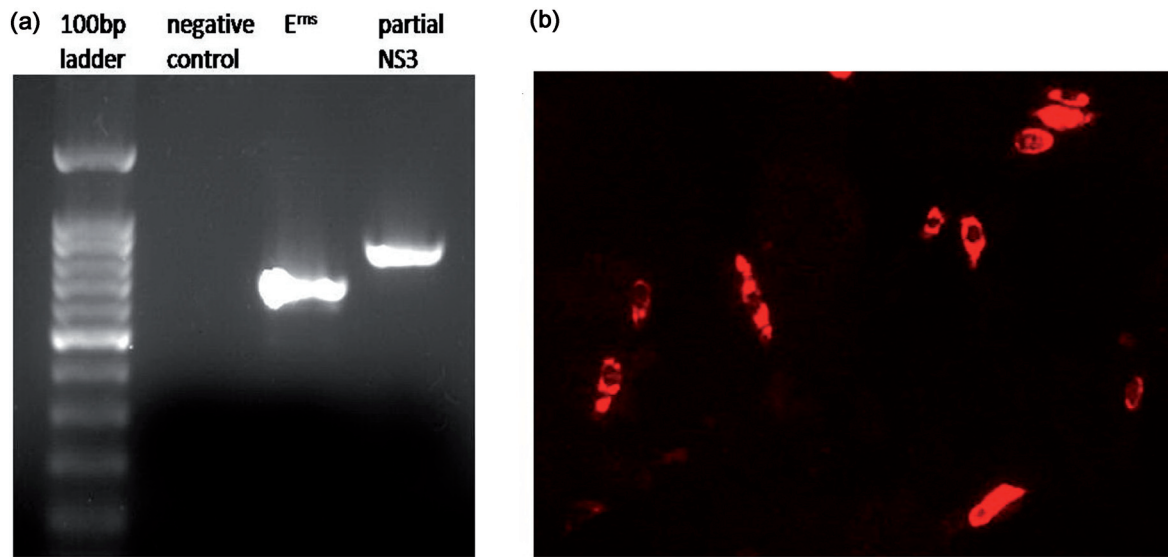


Fig. 2

Identification of chimeric virus vT7SM-c

(a) RT-PCR detecting vT7SM-c in infected cells. (b) Indirect immunofluorescence analysis of vT7SM-c-infected cells using CSFV-specific antibody.

60, and 72 hr post-infection, and viral titers in the supernatant were determined and expressed as PFU/ml in PK15 cells.

Results

Construction of chimeric full-length cDNA clone

The chimeric full-length cDNA clone pT7SM-c was constructed from pT7SM as described in Materials and methods (Fig. 1a) and identified by enzyme digestion (Fig. 1b). The distribution of enzyme restriction sites in pT7SM-c is shown in Table. 1.

Rescue of chimeric virus

RNA transcripts obtained from clone pT7SM and pT7SM-c were used to transfect PK15 cells and the rescued viruses were named vT7SM and vT7SM-c, respectively. The

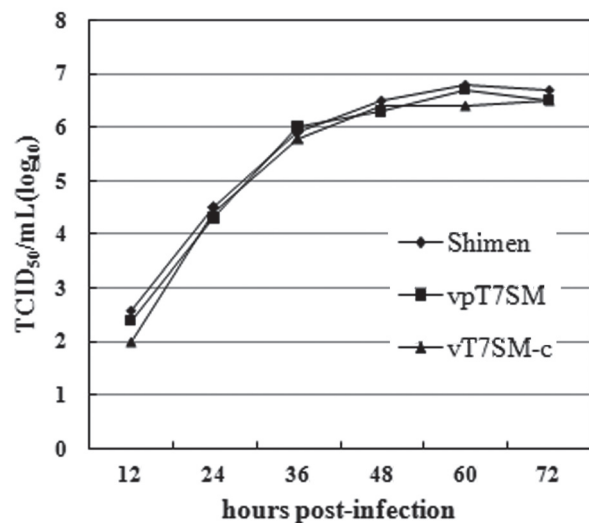


Fig. 3

Growth kinetics of chimeric and parental strains on PK15 cells

cell culture infected by vT7SM and vT7SM-c did not show any cytopathic effect (CPE), which was similar to the infection with Shimen strain (data not shown). After five passages, viral genetic fragments of E^{rns} and partial non-structural NS3 were detected by RT-PCR (Fig. 2a) and virus-specific antigen of E^{rns} was detected by indirect immunofluorescence assay (Fig. 2b), demonstrating the infectivity and stability of vT7SM-c.

Growth kinetics of chimeric virus on PK15 cells

One-step growth curve was drawn to compare the growth kinetics of chimeric virus vT7SM-c with that of its parental virus vT7SM and Shimen. As shown in Fig. 3, the growth characteristics of Shimen, vT7SM and vT7SM-c were almost undistinguished.

Discussion

Here, we described the construction of a chimeric infectious cDNA clone of CSFV. Its biological characteristics were evaluated based on *in vitro* growth properties and CPE on PK15 cells.

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. Recently, investigation of the genetic basis and the molecular mechanism of virulence and viral attenuation based on full-length cDNA clones becomes a fascinating focus in CSFV research. Previously, researches have proposed various mechanisms for different virulence determinants of CSFV (La Rocca *et al.*, 2005; Risatti *et al.*, 2005a,b; Hilton *et al.*, 2006; Risatti *et al.*, 2007; Wang *et al.*, 2008).

As reported, abrogating of E^{rns} RNase activity led to CSFV attenuation (Meyers *et al.*, 1999). However, E^{rns} shows RNase activity in both virulent and avirulent CSFV strains, which has not yet demonstrated whether the genetic region of E^{rns} was involved in affecting viral virulence. Sequence alignment analysis reveals that sequence difference exists in Shimen E^{rns} and HCLV E^{rns} (data not shown), so we wonder whether the attenuation of HCLV is a result of E^{rns} mutation. In this study, we constructed a chimeric infectious cDNA full-length clone of CSFV named pT7SM-c by replacing the E^{rns} genomic region of an infectious clone of CSFV Shimen strain, pT7SM, with the same region obtained from HCLV.

We observed that the rescued chimeric virus vT7SM-c showed no CPE on PK15 cells and had similar growth characteristic as its parental virus. Recent studies have revealed several potential pathogenic mechanisms of viruses, including factors that increase viral replication capacity and

suppress host innate immunity, for example, polymerase polymorphisms of influenza virus increased viral polymerase activity and virulence (Gabriel *et al.*, 2014; Rodriguez-Frandsen *et al.*, 2015; Shao *et al.*, 2015). As we know, the vaccine strain HCLV replicates in an almost imperceptible level compared with other virulent strains, such as Shimen. Hence, the down-regulated replication capacity might be an important factor associated with virus virulence. However, no change was observed in replication capacity of vT7SM-c, which means gene mutation found in E^{rns} might not be a necessary determinant to CSFV virulence.

In conclusion, we created vT7SM-c from a chimeric infectious cDNA clone of CSFV and compared its growth properties with that of the parental strain. This clone will provide an opportunity for genetic manipulation of CSFV and studying of the virulence, protein function and pathogenesis of CSFV.

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References

- Becher P, Thiel HJ (2002): Genus Pestivirus (Flaviviridae). In Tidona CA, Darai G (Eds): *The Springer Index of Viruses*. Springer, Heidelberg, pp. 327–331. http://dx.doi.org/10.1007/3-540-31042-8_53
- Bruschke CJ, Hulst MM, Moormann RJ, van Rijn PA, van Oirschot JT (1997): Glycoprotein Erns of pestiviruses induces apoptosis in lymphocytes of several species. *J. Virol.* 71, 6692–6696.
- Chen L, Xia YH, Pan ZS, Zhang CY (2007): Expression and functional characterization of classical swine fever virus Erns protein. *Protein. Expr. Purif.* 55, 379–387. <http://dx.doi.org/10.1016/j.pep.2007.05.003>
- de Smit AJ, van Gennip HG, Miedema GK, van Rijn PA, Terpstra C, Moormann RJ (2000): Recombinant classical swine fever (CSF) viruses derived from the Chinese vaccine strain (C-strain) of CSF virus retain their avirulent and immunogenic characteristics. *Vaccine* 18, 2351–2358. [http://dx.doi.org/10.1016/S0264-410X\(00\)00027-X](http://dx.doi.org/10.1016/S0264-410X(00)00027-X)
- Gabriel G, Fodor E (2014): Molecular determinants of pathogenicity in the polymerase complex. *Curr. Top. Microbiol. Immunol.* 385, 35–60. http://dx.doi.org/10.1007/82_2014_386
- Hilton L, Moganeradj K, Zhang G, Chen YH, Randall RE, McCauley JW, Goodbourn S (2006): The NPro product of bovine viral diarrhoea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. *J. Virol.* 80, 11723–11732. <http://dx.doi.org/10.1128/JVI.01145-06>
- Hulst MM, Panoto FE, Hoekman A, van Gennip HG, Moormann RJ (1998): Inactivation of the RNase activity of glycoprotein E(rns) of classical swine fever virus results in a cytopathogenic virus. *J. Virol.* 72, 151–157.
- La Rocca SA, Herbert RJ, Croke H, Drew TW, Wileman TE, Powell PP (2005): Loss of interferon regulatory factor 3 in cells

- infected with classical swine fever virus involves the N-terminal protease, *Npro*. *J. Virol.* 79, 7239–7247. <http://dx.doi.org/10.1128/JVI.79.11.7239-7247.2005>
- Mayer D, Hofmann MA, Tratschin JD (2004): Attenuation of classical swine fever virus by deletion of the viral N (pro) gene. *Vaccine* 22, 317–328. <http://dx.doi.org/10.1016/j.vaccine.2003.08.006>
- Meyers G, Saalmüller A, Büttner M (1999): Mutations abrogating the RNase activity in glycoprotein E(rns) of the pestivirus classical swine fever virus lead to virus attenuation. *J. Virol.* 73, 10224–10235.
- Meyers G, Thiel HJ, Rümenapf T (1996): Classical swine fever virus: recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic defective interfering particles. *J. Virol.* 70, 1588–1595.
- Park GS, Lim SI, Hong SH, Song JY (2012): Establishment and characterization of an infectious cDNA clone of a classical swine fever virus LOM strain. *J. Vet. Sci.* 13, 81–91. <http://dx.doi.org/10.4142/jvs.2012.13.1.81>
- Risatti GR, Borca MV, Kutish GF, Lu Z, Holinka LG, French RA, Tulman ER, Rock DL (2005a): The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. *J. Virol.* 79, 3787–3796. <http://dx.doi.org/10.1128/JVI.79.6.3787-3796.2005>
- Risatti GR, Holinka LG, Lu Z, Kutish GF, Tulman ER, French RA, Sur JH, Rock DL, Borca MV (2005b): Mutation of E1 glycoprotein of classical swine fever virus affects viral virulence in swine. *Virology* 343, 116–127. <http://dx.doi.org/10.1016/j.virol.2005.08.015>
- Risatti GR, Holinka LG, Fernandez Sainz I, Carrillo C, Lu Z, Borca MV (2007): N-Linked glycosylation status of classical swine fever virus strain Brescia E2 glycoprotein influences virulence in swine. *J. Virol.* 81, 924–933. <http://dx.doi.org/10.1128/JVI.01824-06>
- Rodriguez-Frandsen A, Alfonso R, Nieto A (2015): Influenza virus polymerase: Functions on host range, inhibition of cellular response to infection and pathogenicity. *Virus Res.* 209, 23–38. <http://dx.doi.org/10.1016/j.virusres.2015.03.017>
- Rümenapf T, Unger G, Strauss JH, Thiel HJ (1993): Processing of the envelope glycoproteins of pestiviruses. *J. Virol.* 67, 3288–3294.
- Ruggli N, Tratschin JD, Mittelholzer C, Hofmann MA (1996): Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. *J. Virol.* 70, 3478–3487.
- Shao J, Liang Y, Ly H (2015): Human hemorrhagic Fever causing arenaviruses: molecular mechanisms contributing to virus virulence and disease pathogenesis. *Pathogens* 4, 283–306. <http://dx.doi.org/10.3390/pathogens4020283>
- Thiel HJ, Stark R, Weiland E, Rümenapf T, Meyers G (1991): Hog cholera virus: molecular composition of virions from a pestivirus. *J. Virol.* 65, 4705–4712.
- Wang Y, Wang Q, Lu XL, Zhang CY, Fan XZ, Pan ZS, Xu L, Wen GY, Ning YB, Tang FQ, Xia YH (2008): 12-nt insertion in 3' untranslated region leads to attenuation of classic swine fever virus and protects host against lethal challenge. *Virology* 374, 390–398. <http://dx.doi.org/10.1016/j.virol.2008.01.008>
- Wu HX, Zhang CY, Zheng CY, Wang JF, Pan ZS, Li L, Cao S, Yi GH (2003): Construction of cytopathic PK15 cell model of classical swine fever virus. *Chin. Sci. Bull.* 48, 887–891. <http://dx.doi.org/10.1360/02wc0437>