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Original Article

Establishment and characterization of an infectious cDNA clone of a classical swine fever virus LOM strain

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Classical swine fever virus (CSFV) causes a highly contagious disease among swine that has an important economic impact worldwide. CSFV strain LOM is an attenuated virus of low virulent strain of Miyagi isolated from Japan in 1956. Eight DNA fragments representing the genome of the CSFV strain LOM were obtained by RT-PCR. These were used to determine the complete nucleotide sequence and construct a full-length cDNA clone which was called Flc-LOM. Sequence analysis of the recombinant clone (Flc-LOM) revealed the presence of eight mutations, resulting in two amino acid substitutions, when compared to the parental sequence. RNA transcripts of both LOM and Flc-LOM were directly infectious in PK-15 cells. The rescued Flc-LOM virus grew more slowly than the parental virus, LOM, in the cells. Intramuscular immunization with Flc-LOM was safe and highly immunogenic in pigs; no clinical signs or virus transmission to sentinel animals were observed after 35 days. CSFV-specific neutralizing antibodies were detected 14 days post-infection. After challenge with the virulent CSFV strain SW03, pigs immunized with Flc-LOM were shown to be fully protected. Thus, our newly established infectious clone of CSFV, Flc-LOM, could serve as a vaccine candidate.

Keywords: classical swine fever virus, Flc-LOM, infectious c DNA clone, LOM

Introduction

Classical swine fever virus (CSFV) is the etiological agent of classical swine fever, a devastating disease of swine and wild boar also known as hog cholera. This disease can have an acute or chronic course and causes significant economic losses in the pig industry worldwide [6]. Together with bovine viral diarrhea virus (BVDV) and border disease virus, CSFV belongs to the genus *Pestivirus* within the *Flaviviridae* family [29]. The spherical virions are 40～60 nm in diameter. The genome is not segmented and contains a single positive-sense RNA strand about 12.3 kb in length. The 5′ and 3′ ends of the genome do not have a cap or poly (A) tract [17]. CSFV proteins are translated from genomic RNA as a single large polyprotein of about 3,900 amino acids, which is co- and post-translationally processed by cellular and viral proteases to form mature viral proteins [23,33].

Two main strategies for controlling CSF, depending on the epidemiological conditions of the affected geographical area, are systematic prophylactic vaccination with a live attenuated vaccine and a non-vaccination, stamping-out policy. The former technique makes it impossible to distinguish naturally infected from vaccinated animals, and the latter carries a risk of enormous outbreaks which can result in huge economic losses in areas with a high density of pigs [7]. Although potentially serious side effects such as pig chromosome aberrations exist after vaccination with a live attenuated vaccine [9], vaccination has been the most effective tool for preventing CSF. For eradication and control purposes, available CSFV vaccines are based on the Chinese (C) strain, cell culture-adapted Japanese guinea pig exaltation-negative (GPE^-) strain, or French cell culture-adapted Thiverval strain and its derivatives [28]. These vaccines, which have been proven to be efficacious and safe, are obtained after serial passage of CSFV isolates in tissue cultures or rabbits, and the genetic basis of their attenuation is unknown.

The LOM strain, an attenuated virus of low virulent strain of Miyagi isolate from Japan, has been used as a live

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attenuated CSF vaccine in Korea for more than 30 years [11]. Several studies have described the use of the attenuated LOM virus as a vaccine strain. This strain was first isolated by Sato *et al.* [26] in 1956 from naturally infected swine in the Miyagi Prefecture (Japan) and was further attenuated in their laboratory by continuous propagation in bovine kidney cells. A bovine kidney tissue culture-attenuated live vaccine, the LOM strain, was established by the National Veterinary Assay Laboratory in Japan in 1964 [20]. The LOM strain was cloned, attenuated, and then tested as vaccine candidate from 1968 to 1969 in the field by Istitute of Veterinary Research (IVR) in Korea [11,12]. The strain has been used as a live vaccine to eradicate CSFV in the field since 1974, and was shown to be safe and highly immunogenic in pigs for many decades [13].

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. As with many other viruses, the generation and use of infectious CSFV clones have provided new opportunities for understanding and characterizing the mechanisms of viral replication and pathogenesis [21]. Information generated using reverse genetics has enabled the identification of CSFV proteins or protein domains that determine virulence and host range, as well as facilitating the rational design and development of new vaccines against CSF. To date, all infectious cDNA clones that are routinely used for molecular studies have been established from the moderately virulent Alfort/187 strain of CSFV [22]; the C-strain, which is the most well-known and widely used attenuated vaccine strain in the world [19]; the highly virulent Eystrup strain; and the avirulent Riems/IVI vaccine strain [16]. 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 [18, 19,22,30]. 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.

The objectives of the present study were to construct a full-length cDNA clone of the original attenuated CSFV strain LOM, and to compare the characteristic features of the parental and recombinant (Flc-LOM) strains including safety, efficacy, and *in vitro* and *in vivo* utility. An infectious cDNA clone of the CSFV LOM strain, Flc-LOM, was generated. We showed that pigs immunized with Flc-LOM were fully protected against CSFV infection when exposed to the virulent SW03 strain. Establishment of infectious clones derived from a live attenuated CSFV vaccine strain should significantly facilitate ongoing studies to determine the molecular basis of virulence and attenuation as well as enabling accurate genomic manipulation. Furthermore, these can serve as valuable tools for developing new potent marker vaccine.

Materials and Methods

Cells and viruses

The porcine kidney cell line PK-15 (ATCC, USA) was maintained in α-minimal essential medium (α-EMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA) which has no antibody against BVDV at 37°C in a humidified atmosphere of 5% CO_2. The LOM strain (accession No. EU789580, GenBank) was developed as a CSFV vaccine and was distributed to five Korean animal vaccine companies by the Animal, Plant and Fisheries Quarantine and Inspection Agency, Korea.

The nucleotide sequence of Flc-LOM rescued from a full-length infectious clone has been submitted to GenBank (accession No. EU915211). The virulent CSFV strain SW03 was previously obtained from field isolates in Korea [2,31]. Virus stocks were prepared by several passages in PK-15 cells. Virus preparations were tested for the absence of BVDV, porcine circovirus-2, and mycoplasma by polymerase chain reaction (PCR) using specific primers against them.

Recombinant DNA procedures

Escherichia coli XL-1 Blue (Stratagene, USA) was grown at 37°C in Luria-Bertani medium (Becton Dickinson, USA) supplemented with ampicillin (100 μg/mL; Sigma, USA). Standard recombinant DNA techniques were performed as described by Sambrook and Russell [25]. Restriction endonucleases, *Sac*II, *Hid*III, *NgoM*IV, *Kpn*I, *Ssp*I, were purchased from New England Biolabs (USA) and *Srf* I from Stratagene (USA). For PCR, AccuPrime Pfx DNA polymerase (Invitrogen, USA) was used as recommended by the manufacturer. DNA fragments were separated and isolated from agarose gels using a MinElute kit (Qiagen, Germany).

Extraction of viral RNA and RT-PCR

PK-15 cells were infected with parental LOM virus at a multiplicity of infection (MOI) of 1, and the culture supernatant was collected at 72 h post-infection (p.i.). The culture supernatant was clarified by centrifugation at 1,000 × g for 10 min; RNA was extracted with an RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's recommendations. The viral RNA was resuspended in nuclease-free water. cDNA was synthesized using Superscript III (Invitrogen, USA) with specific primer sets to generate eight individual PCR fragments (designated amplicons 1 ~ 8, Table 1) according to the manufacturer's recommen-

Table 1. Primers used for RT-PCR to synthesize eight overlapping cDNA clones for creating Flc-LOM

Primer name	Nucleotide sequence	Location within LOM genome
Amplicon 1		
XT7-KLOM	5′-CTACTCGAGTTAATACGACTCACTATAGTATACGAGGTTAGCTC-3′	1～17
KLOM-5	5′-GTCTGGCATTCTCTATAGTGTTG-3′	1,735～1,757
Amplicon 2		
KLOM-27	5′-GAATACCCAAGACGGCCTGTACCAC-3′	1,054～1,078
KLOM-28	5′-GGAACCTGCCACGCAAATGGCCTTA-3′	2,572～2,596
Amplicon 3		
KLOM-29	5′-GTTATTTGAAGAGGCAGGACAGGTC-3′	2,266～2,290
KLOM-16	5′-CAATGGCAACATGGTACCTGTTATTG-3′	4,440～4,465
Amplicon 4		
KLOM-14	5′-GTACGGATATAGCCATAGCTAC-3′	3,942～3,963
KLOM-2	5′-GACCGACCTAGGAAGTTCCGTG-3′	5,839～5,860
Amplicon 5		
KLOM-20	5′-CTACAACTCAGGCTACTATTATAG-3′	6,406～6,429
KLOM-25	5′-GACCAGAGTGGAGTGATCAACCTC-3′	8,852～8,875
Amplicon 6		
KLOM-8	5′-GAAGCAGTGCAGACAGTCGGCAACCCTC-3′	8,240～8,767
KLOM-18	5′-CAGATTGTCAATAATCTCTTCGAC-3′	10,634～10,657
Amplicon 7		
KLOM-19	5′-GAGAGGAATAAACAGGAAGGGTG-3′	10,552～10,577
KLOM-4	5′-GCTACTCACTAGTCTGTCTGC-3′	11,858～11,878
Amplicon 8		
KLOM-1	5′-CA ACCACAATCC TGGCTAAAAT GGCCACAAG-3′	11,469～11,499
BS-KLOM	5′-TAGGATCCGCCCGGGCCGTTAGGAAATTAC-3′	12,281～12,298

dations. PCR was carried out using Pfx DNA polymerase (Invitrogen, USA) for higher DNA synthesis fidelity. Briefly PCR mixture was made by mixing 5 μL of 10 × Pfx DNA polymerase buffer, 1.5 μL of each 10 μM of primer mix, 200 ng of template DNA, 2.5 U of Pfx DNA polymerase and adjust to 50 μL with autoclaved distilled water. Denature the mixture for 2 min at 95°C and anneal at room temperature for 5～10 min. PCR amplication cycles as follows: denaturaton at 95°C for 15 sec, annealing at 55～64°C for 30 sec and extension at 68°C for 2～3 min and a single 5 min extension at 68°C.

Construction of Flc-LOM

To generate a full-length infectious cDNA clone of LOM, we used a modified methods previously described by Meyer *et al.* [16]. To assemble a single clone, the eight overlapping cDNA amplicons were inserted into a pACYC177 plasmid (New England Biolabs, USA), in which the kanamycin resistance site completely deleted, using available restriction sites in the amplicons along with *Sac*II and *Srf* I sites in the vector (Fig. 1A, left panel). First, the 5′ terminal amplicon 1 (1～1,256 bp) of the viral genome was modified so that it included a T7 promoter and unique *Sac*II site, derived from pGEM-T Easy (Promega, USA), for insertion. The 3′ terminal amplicon 8 (11,729～12,298 bp) was inserted into the unique *Srf*I site to yield the authentic viral genome (Fig. 1A, right panel). The modified 5′ and 3′ end fragments in pACYC177 were digested with *Hind*III and *Ngo*MIV, and subsequently ligated to amplicon 2 (1,256～2,440 bp). Using this construct, we continued to insert amplicons into the plasmid. For example, amplicon 3 (2,440～4,453 bp) was digested with *Ngo*MIV and *Kpn*I, and ligated into the restriction site of amplicon 2. Amplicons 4, 5, and 7 were similarly assembled into the appropriate restriction sites. Finally, *Ssp*I-digested amplicon 6 (8,833～10,612 bp) was ligated into the *Ssp*I site, generating the full-length cDNA clone pFlc-LOM (Fig. 1A).

In vitro synthesis of viral RNA, transfection of PK-15 cells, and rescue of Flc-LOM

The full-length cDNA clones of LOM were linearized with the restriction endonuclease *Srf*I, which cut at the 3′ end of the viral genome. The template DNA was treated with proteinase K (200 μg/mL; Promega, USA) to remove

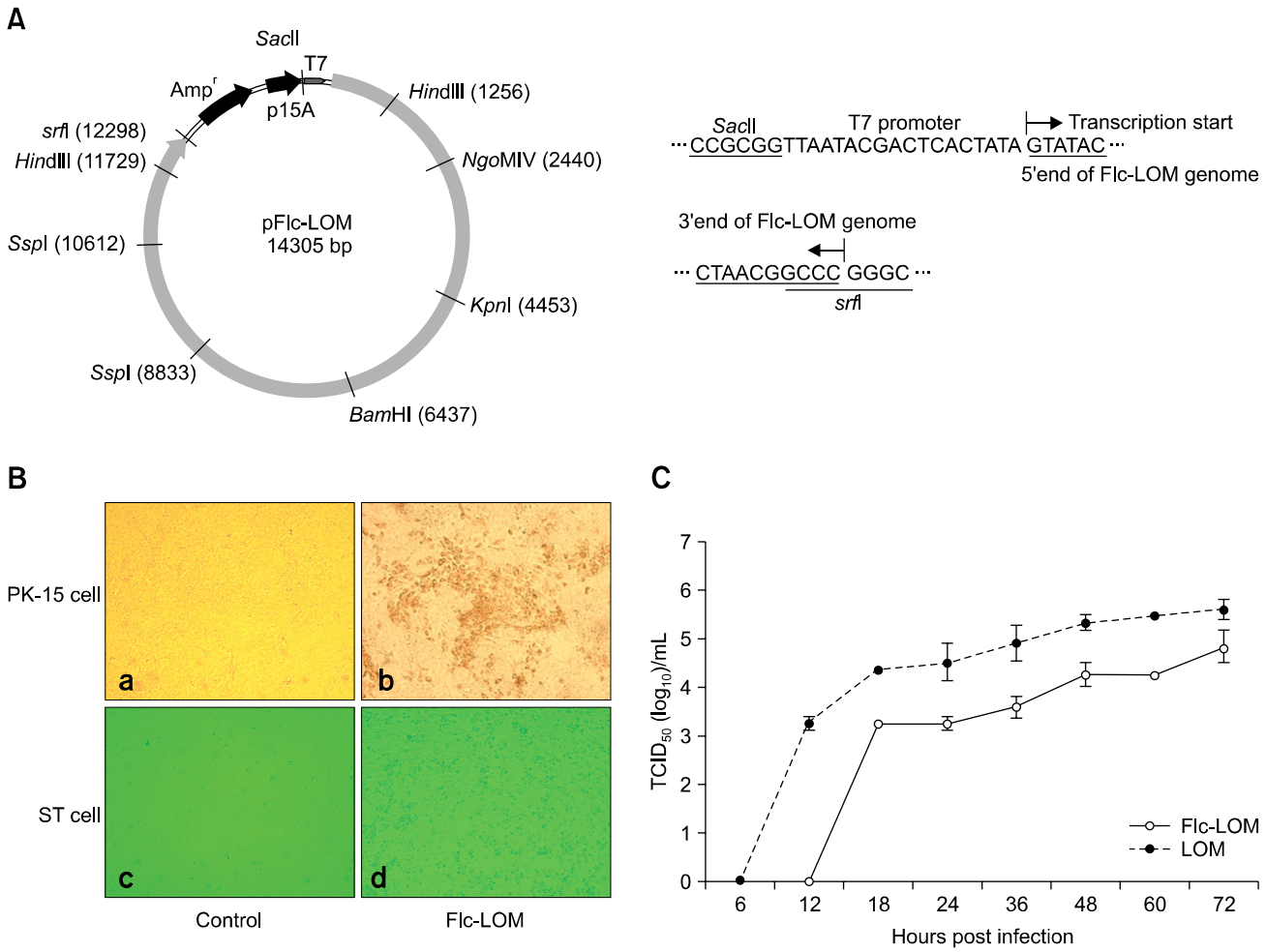

Fig. 1. Strategies for construction and *in vitro* growth characterization of Flc-LOM. (A) Circular map of pFlc-LOM showing the relative positions of the LOM cDNA, ampicillin resistance gene, and P15A origin of replication. Locations of relevant restriction sites and the T7 promoter are indicated. The T7 RNA promoter was located immediately upstream of the 5′ end of the genome, and a *Srf*I site was at the 3′ end. The genome sequences are underlined. (B) Using the classical swine fever virus-specific monoclonal antibody LOM01, an indirect immunoperoxidase assay of Flc-LOM was performed in PK-15 cells 3 days after infection with a multiplicity of infection (MOI) of 1 (a and b, × 200). The Flc-LOM biotype was determined using the exaltation of Newcastle disease virus method. Serial dilutions from $10^{1.0}$ to $10^{6.0}$ of Flc-LOM were added to primary swine testicular cells. After 4 days, the supernatant was removed and the cells were infected with Newcastle disease virus ($10^{4.0}$ PFU/mL). A cytopathic effect was observed 3 days post-infection and photographed using an inverted microscope (c and d, × 200). (C) Replication kinetics of the Flc-LOM and LOM viruses. PK-15 cells seeded in T25 flasks were infected at a MOI of 1. The data are from three independent experiments. Virus titers are shown as $\log_{10} 10^4$ median $TCID_{50}$/mL. ST: swine testicular.

contaminating RNAase A, extracted with phenol/chloroform, and precipitated with ethanol. Authentic viral RNA was obtained by transcription from the T7 RNA polymerase promoter using a T7 MEGAscript kit (Ambion, USA). After TURBO DNase I (Ambion, USA) digestion, the transcripts were purified by lithium chloride precipitation and quantified spectrophotometrically by spectrophotometer (ND - 1000; Nano Drop Technologies, USA). *In vitro*-synthesized RNA (1.5 μg) and lipofectin reagent (Invitrogen, USA) were used to transfect 2×10^5 PK-15cells which were grown in 6-well tissue cultue plates (TPP, Switzerland). Culture supernatants were collected 3 days after transfection, and 1 mL of culture supernatants were inoculated into the monolayers of PK-15 cells to determine the presence of infectious virus by immunocytochemical staining [8]. The culture supernatants with virus were used for virus passage for amplification, determination of replication kinetics, and experimental infection of pigs.

In vitro characterization of Flc-LOM

The viruses were characterized by an indirect immunoperoxidase assay. PK-15 cells were infected with a 10-fold dilution of Flc-LOM, and the titer was determined 48 h later by immunocytochemical staining the cells with the

monoclonal antibody LOM01 (Jeno Biotech, Korea) directed against glycoprotein E2 [8]. The enhancing effect of CSFV on Newcastle disease virus (NDV) multiplication in primary swine testicular cells, known as exaltation of Newcastle disease (END) virus method, was evaluated as previously described [14]. Primary swine testicular cells were prepared from testicls removed aseptically from a 10 kg of landlace pig, trypsinized, and 100 μL of 1×10^5 cells were transferred to each well of 96-well tissue cluture plates (TPP, Switzerland). Serial dilutions from 10^{-1} to 10^{-8} of Flc-LOM (0.1 mL) were added to each well, and the cells were infected for 2 h at 37°C. The α-EMEM (Invitrogen, USA) with 10% FBS(Invitrogen, USA) was changed after 72 h, and the plates were inoculated with NDV Miyadera strain (10^6 PFU/mL). After 72 h of incubation, we observed cytopathic effects. The growth kinetics of Flc-LOM and parental LOM were determined in PK-15 cells. Subconfluent monolayers in T25 flasks (TPP, Switzerland) were infected at an MOI of 1. Cells were incubated for 1 h at 37°C, washed, and then incubated in fresh α-EMEM (Invitrogen, USA). At the indicated times p.i., the respective cell cultures were frozen to stop virus replication, and the virus was titrated in PK-15 cells as follow. Serial dilutions from 10^{-1} to 10^{-8} of Flc-LOM (0.1 mL) were added to 96 well tissue culture plate. Add to all wells 50 μL of growth media containg 2×10^5 cells. Titer was determined 48 h later by immunocytochemical staining the cells with the monoclonal antibody LOM01(Jeno Biotech, Korea) directed against glycoprotein E2 [8].

Animal experiments

Eighteen 45-day-old specific pathogen-free piglets with a certified record of the absence of CSFV infection were purchased from Optifam Solution (Korea). The animals were divided into three groups: two experimental groups (n = seven animals per group) and one control group (n = 4). In each of the two experimental groups, five piglets were inoculated intramuscularly with parental LOM virus or full-length cDNA-derived Flc-LOM. Inoculation dose of Flc-LOM and LOM virus was 10^4 median tissue culture-infective doses/mL ($TCID_{50}$/mL) in a volume of 1 mL; two piglets in each group were designated as sentinels. The four control piglets were inoculated with 1 mL of α-EMEM (Invitrogen, USA) with 5% FBS (Invitrogen, USA). After 35 days, three piglets in the Flc-LOM and LOM groups and four in the control group were challenged with SW03, a virulent CSFV strain, at a virus concentration of 100 LD_{50} by intramuscular inoculation. During the course of the experiment, the piglets were monitored daily for clinical signs (coughing, sneezing, appetite loss, and movement) and rectal temperature was measured. Blood samples along with fecal and nasal swabs were taken for virological and serological examination before and after primary inoculation and challenge. Blood samples were also used to determine leukocyte counts. Twenty-eight days after the challenge, the piglets inoculated with Flc-LOM or LOM and the control piglets were euthanized. Blood and tissue samples from tonsil, spleen, liver, lung, heart, kidney and mesenteric lymphonode were collected for virological examination. Virus was isolated from blood and tissue samples by co-cultivation with PK-15 cells for 4 days. After incubation, the cells were analyzed for detection of E2 antigen by immunofluorescence assay with the monoclonal antibody LOM01 (Jeno Biotech, Korea). To detect CSF-specific neutralizing antibodies, a neutralizing peroxidase-linked assay was performed according to the standards manual of the World Organization for Animal Health [8]. Neutralizing antibody titers were expressed as reciprocal numbers of the highest two-fold serum dilution. Two-fold dilutions of serum starting at 1 : 2 were prepared for this experiment. Serum dilutions higher than eight were positive.

Results

Sequencing and cloning of the full-length genomic RNA of CSFV LOM

The full-length genomic sequence of the CSFV LOM strain was determined. Eight overlapping cDNA fragments were generated by RT-PCR using various PCR primers (Table 1). All regions of the genome were sequenced by at least three independent RT-PCR procedures. The genome of the virus was determined to be 12,298 nucleotides in length. Based on a comparison of the complete nucleotide sequence of the CSFV LOM strain with sequences found in GenBank, LOM was found to be most closely related (with 99% nucleotide sequence identity) to the CSFV strain Alfort/187 (GenBank No. X87939.1) and Pestivirus type 2 strain Alfort A19 (GenBank No. U90951.1), a moderately virulent strain. The LOM sequence also showed 98% identity with the published sequences for the vaccine referred to as Thiverval and with GPE^- strains (GenBank Nos. EU490425.1 and D49533.1, respectively).

When each of the eight PCR amplicons, representing different regions of the LOM genome, was subcloned and sequenced, eight nucleotide alterations were observed (Table 2). Two mutations, C to T at nucleotide position 52 of the genome and A to G at position 96, were in the non-translated region. Two mutations, G405A and G6049A, resulted in the substitution of glycine for glutamic acid and methionine for isoleucine, respectively. The other four changes were silent mutations. Nearly all of the amplicons deviated from the consensus sequence by at least one base substitution, presumably due to PCR-induced mutations; however, the resultant amino acid changes had no effect on the recovery of infectious clones and thus may serve as genetic markers.

Table 2. Nucleotide and predicted amino acid differences between LOM and Flc-LOM

Nucleotide position	Nucleotide in LOM*	Nucleotide in Flc-LOM†	Amino acid change	Genome position
52	C	T		NTR
96	A	G		NTR
405	G	A	G → E	N^{pro}
2608	A	G	Silent	E2
5899	A	G	Silent	NS3
5956	G	A	Silent	NS3
6049	G	A	M → I	NS3
7054	G	T	Silent	NS4A

*,†Nucelotide positions within the LOM and Flc-LOM genome are based on GenBank accession Nos. EU789580 and EU915211, respectively.

Construction and recovery of infectious Flc-LOM

To assemble the eight overlapping cDNA fragments into a single clone, the amplicons were sequentially ligated into the low copy-number plasmid pACYC177 after the plasmid was cleaved with the following enzymes: *Hind*III at nucleotide position 1,256; *Ngo*MIV at 2,440; *Kpn*I at 4,453; *Bam*HI at 6,437; *Ssp*I at 8,833 and 10,612; *Hind*III at 11,729; and *Srf*I at 12,298 (Fig. 1A, left panel). The 5′ end of the genome included a *Sac*II restriction site and the T7 promoter sequence immediately upstream of the viral genome for *in vitro* transcription of authentic viral RNA. At the 3′ end of the viral genome, a *Srf*I restriction site was introduced to allow the generation of authentic viral RNA by runoff transcription (Fig. 1A, right panel). The assembled full-length cDNA clone, called pFlc-LOM, was linearized by digestion with *Srf*I and used as a template for *in vitro* transcription by T7 RNA polymerase.

PK-15 cells were transfected with the *in vitro* transcripts by liposome-mediated transfection. About 50% of the cells expressed envelope glycoprotein E2 at 72 h post-transfection. Supernatants collected from the transfected cells contained infectious CSFV as demonstrated by the addition of supernatant to PK-15 cells. Culture supernatant from the first passage was recovered at 3 days p.i. and consistently yielded a viral titer of $10^{5.5}$ $TCID_{50}$/mL. To confirm that the virus was recovered from cells infected with Flc-LOM transcripts, we used an immunoperoxidase assay with the monoclonal antibody LOM01 raised against glycoprotein E2 of CSFV. The cytoplasm-specific staining pattern of Flc-LOM-infected cells was identical to that of cells infected with the parental LOM virus (Fig. 1B, upper panel). Nucleotide sequencing results confirmed that the rescued virus maintained the Flc-LOM nucleotide changes (Table 2).

In vitro growth properties of Flc-LOM

Pestiviruses are divided into two biotypes, cytopathogenic and non-cytopathogenic, based on the resulting distinguishable morphological changes in cultured cells. We tested the END phenomenon in swine testicular cell cultures. Flc-LOM stimulated NDV cytopathogenicity in primary swine testicular cells, indicating that one biological phenotype of Flc-LOM was END phenomenon-positive (Fig. 1B, lower panel). An analysis of the replication kinetics of Flc-LOM and parental LOM virus in PK-15 cells revealed that the LOM virus grew faster and reached a higher titer (by about 1 log unit) than the Flc-LOM virus at different time points (Fig. 1C).

In vivo properties of Flc-LOM in pigs

After immunizing pigs with the Flc-LOM or parental LOM virus, the general condition of the animals was normal, irrespective of the inoculum, and no clinical signs, leukopenia, or fever were observed. Blood leukocytes from all vaccinated animals were positive for the virus, as determined by RT-PCR, 5～7 days after immunization. In several immunized piglets, viral antigens were detected by RT-PCR in fecal and nasal swabs 5～7 days p.i. (Table 3). None of the sentinel animals in the immunized groups showed increased body temperature or leukopenia, and no viral antigen was detected in their blood, fecal, or nasal swabs. RT-PCR was used to identify viral antigens in tissue samples after immunization and challenge (Table 4). After immunization or challenge, viral antigens were detected from tonsil, lung, spleen, liver, and mesenteric lymph node of pigs in LOM group, but only from the tonsil in Flc-LOM group was detected. However, viral antigens from all tissue samples in control group were detected. Serological screening using a LOM-specific neutralization assay demonstrated that none of the Flc-LOM or LOM sentinel animals had seroconverted (data not shown). Although Flc-LOM viremia was detected at an early stage, shedding of the virus was not observed in the sentinel animals. On day 28 p.i., the animals immunized with Flc-LOM and LOM strains had CSF-neutralizing antibody titers higher than 8, whereas none of the non-immunized animals had CSFV-E2- or LOM-specific serum antibodies (Table 5).

After intramuscular challenge with the virulent CSFV strain SW03 at 35 days after immunization, the health of the pigs previously inoculated with Flc-LOM or LOM remained completely unchanged, and neither fever nor leukopenia was detected in any of the animals (Fig. 2). In contrast, all the control pigs became anorectic and severely depressed after CSFV SW03 inoculation. Typical CSF symptoms started 3 days after inoculation and lasted for 14～21 days. Severe leukopenia occurred between days 3 and 21 post-challenge, and fever with body temperatures higher than 41.5°C lasted for several days in the control pigs (Fig. 2). These results indicate that, like the pigs

Table 3. Detection of viral antigen (in blood, fecal, and nasal swabs) by RT-PCR following immunization (Flc-LOM and LOM) or challenge infection (control)

Sample	Group	Days after 1st immunization (days after 2nd immunization)												Days after challenge infection					
		0	3	5	7	10	14	21	24 (3)	26 (5)	28 (7)	31 (10)	35 (14)	3 38	5 40	7 42	10 45	14 49	21 56
Blood	Flc-LOM	0/5*	0/5	5/5 (2)†	5/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/3	0/3	0/3	0/3	0/3	0/3
	LOM	0/5	0/5	5/5 (1)	4/4 (2)	2/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1/3	2/3	0/3	2/3	0/3	0/3
	Control	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	4/4	4/4	4/4	4/4	4/4
Fecal swab	Flc-LOM	0/5	0/5	1/5	2/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/3	0/3	0/3	0/3	0/3	0/3
	LOM	0/5	0/5	1/5	1/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/3	0/3	0/3	1/3	0/3	0/3
	Control	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	4/4	4/4	4/4	4/4	4/4
Nasal swab	Flc-LOM	0/5	0/5	1/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/3	0/3	0/3	0/3	0/3	0/3
	LOM	0/5	0/5	3/5	4/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/3	0/3	0/3	0/3	0/3	0/3
	Control	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	4/4	4/4	4/4	4/4

*The number of positive/number of tested piglets. †The number of virus isolation from positive samples by RT-PCR.

Table 4. RT-PCR detection of viral antigens in tissue samples after immunization or challenge infection

Group	The number of positive/number of tested pig										
	Tonsil	Lung	Heart	Spleen	Liver	Kidney	Mesenteric lymph node	Ileum	Ceucum	Inguinal lymph node	Brain
Flc-LOM	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
LOM	2/2	1/2	0/2	1/2	1/2	0/2	1/2	0/2	0/2	0/2	0/2
Control	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	0/4

Table 5. Mean neutralizing antibody titers (log2) in pigs from the Flc-LOM, LOM, and control groups after immunization or challenge infection

Group	Days after 1st immunization (days after 2nd immunization)						Days after challenge infection		
							7	14	21
	0	7	14	21	28 (7)	35 (14)	42	49	56
Flc-LOM	0.0	0.8	5.8	7.0	8.3	8.5	10.3	11.0	12.0
LOM	0.0	0.8	5.3	6.3	8.0	8.8	10.0	11.0	12.0
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0

immunized with parental LOM, the Flc-LOM-immunized pigs were completely protected against challenge with the virulent SW03 strain. Overall, the infectious Flc-LOM virus cloned from the LOM strain of CSFV retained the biological and immunogenic properties of the parental virus.

Discussion

Here, we described the construction of an infectious cDNA clone of the CSFV LOM strain. Its biological characteristics were evaluated based on *in vitro* growth properties and the resulting END phenomenon in primary ST cells. We also demonstrated the utility of Flc-LOM as a vaccine candidate by investigating the *in vivo* phenotype and immunogenicity of this construct in a pig model. This study is the first to perform the construction of a full-length LOM strain. This clone will prove valuable for studying the replication, virulence, cell and host tropism, and pathogenesis of CSFV.

Reverse genetics is a useful tool for studying viral replication, pathogenesis, and the *in vivo* function of individual viral proteins as well as developing viruses as vectors and vaccines. Reverse genetics allows the introduction of changes at specific sites or regions of the viral genome to create modified infectious viruses [15]. This technology is particularly useful for RNA viruses because their genomes are unstable. The construction of infectious cDNA clones of several CSFV strains has been previously reported [22].

In Korea, an attenuated live vaccine (LOM strain) has been used extensively since 1974 to control CSF in the field. Recently, some pig farmers and veterinarians have suspected that the strain may have mutated and became pathogenic [13]. The LOM strain has been maintained through a number of passages in bovine/porcine kidney cells for several years. Although this strain is considered genetically stable, its reversion to a virulent strain seems be possible. For this reason, we established Flc-LOM to analysis the pathogenesis of the LOM strain. The LOM

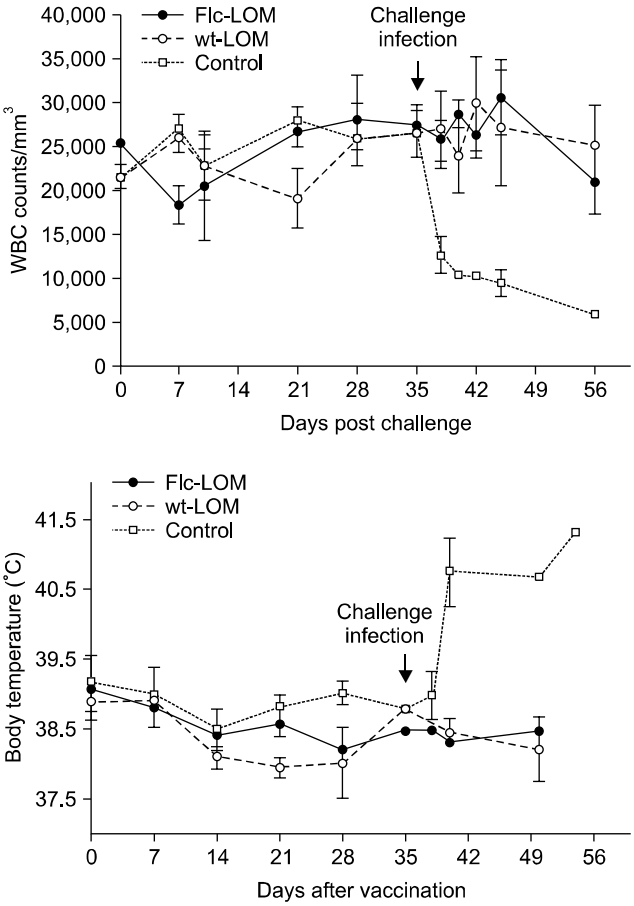

Fig. 2. Evaluation of clinical responses in pigs infected with Flc-LOM or LOM. CSFV-specific immunity was determined on day 35 p.i. with the virulent CSFV strain SW03. (A) Peripheral white blood cell (WBC) counts in pigs infected with Flc-LOM or LOM. Mean values of the leukocyte counts of the Flc-LOM (n = 5), LOM (n = 5), and control groups (n = 5) are shown. Standard deviations are expressed as error bars (B). Body temperatures were recorded for the Flc-LOM, LOM, and non-vaccinated control groups until 2 weeks after challenge.

strain is an effective and safe live vaccine. However, pigs that are vaccinated with the LOM strain cannot be serologically differentiated from pigs which have been

infected with a CSFV field strain. The Flc-LOM clone created in our study will provide the basis for constructing chimeric viruses and marker vaccine.

To establish authentic cDNA clones of the LOM strain, the full-length genomic RNA sequence was determined. To ensure the authenticity of the synthesized RNA, the clone sequence (Flc-LOM) was placed downstream of a T7 promoter, and a *Srf*I site was introduced at its 3′ end. Full-length genomic RNA was synthesized *in vitro*, and the RNA transcripts were used for transfection. The expression of E2 proteins appeared as early as 2 days post-transfection and infectious Flc-LOM virus was readily recovered. The recovered Flc-LOM virus was stable for several passages with a titer of $>10^{5.5}$ $TCID_{50}$/mL (data not shown). The titer of Flc-LOM in PK-15 cells was reduced by only about 1 log unit compared to that of the parental LOM strain. We speculate that amino acid or nucleotide differences between the Flc-LOM and parental virus were probably responsible for inefficient Flc-LOM growth in PK-15 cells. Establishment of a pathogenic infectious cDNA clone will benefit investigations of certain genes associated with the pathogenesis and virulence of CSFV.

The END phenomenon is based on the enhancing effect of CSFV on NDV in swine testicular cells [14], which is one CSFV characteristic. Sato *et al.* [26] previously reported that LOM strains was not detected by the cytopathic effect in the END test. But it was reported that LOM vaccine used in Korea was mixed with two types of viruses showing END phenomenon-negative or END phenomenon-positive [3]. Others also reported that low-virulence strains of hepatitis C virus in Germany could not be detected by the END method [20]. Most CSFV field isolates that show the END phenomenon do not induce a cytopathic effect in porcine cell cultures [14]. The Japanese live vaccine GPE^- strains prepared by long-term serial passage are END phenomenon-negative. To date, there have been no reports of END phenomenon-negative viruses being isolated independently from animals suffering from CSF in field outbreaks [1].

Although Flc-LOM was shown to be non-cytopathogenic but END phenomenon-positive, the efficacy and safety of Flc-LOM as a vaccine candidate were demonstrated. These results implied that there is no direct relationship between the virulence of CSFV and the END phenomenon. This suggests that our Flc-LOM strain was selected as END phenomenon-positive strain in contrast to the LOM strain of Sakoda *et al.* [24] which is END phenomenon-negative. Difference in the presence of the END phenomenon might have occurred because the LOM strain of Sato *et al.* [26] is an earlier isolate and may contain a heterogeneous population with END phenomenon-negative virus or END phenomenon-positive virus. Further investigation is necessary to determine the phenotype of END phenomenon-negative virus and genetical characterization of this virus.

To date, all live and modified live vaccine candidates [4,27,32] that express CSFV structural proteins need to be delivered in multiple or at very high doses to induce clinical protection. In our animal experiments, the newly established live vaccine Flc-LOM provided complete protection from virulent CSFV challenge after only a single application. Flc-LOM-infected animals did not develop a fever and maintained body temperatures at pre-challenge levels. In contrast, the control pigs had severe forms of the disease and had to be euthanized. Leukocyte counts in Flc-LOM-infected pigs remained stable. Although shed Flc-LOM virus was undetectable in nasal and fecal swabs, some virus was detected in blood samples. The detection of viral genomes has been described to be more sensitive than conventional virus isolation, especially in the presence of neutralizing antibodies [10]. Nevertheless, amplification of the Flc-LOM genome in nasal and fecal swabs was not successful. The detection of viral antigen by RT-PCR suggested that Flc-LOM was attenuated to a greater extent than parental LOM virus in terms of *in vivo* replication efficacy. Non-vaccinated sentinel animals did not seroconvert against Flc-LOM or exhibit signs of disease. Therefore, we speculate that Flc-LOM is highly effective in reducing or preventing transmission of CSF virus in the field. A major safety aspect concerning vertical transmission in pregnant sows is currently under investigation.

Flc-LOM, the infectious clone of CSFV LOM, proved to be as avirulent as the parental LOM strain. This clone did not cause any disease symptoms, but induced a distinct antibody response and provided complete protection from challenge with the virulent strain SW03. Similarly, de Smit *et al.* [5] have reported a cDNA clone derived from the C strain, which is the most widely used vaccine, that also retains its parental phenotypic characteristics, including antigenic and immunogenic properties. Production of a rationally designed live attenuated vaccine will be valuable for disease control strategies in the event of a future CSFV outbreak.

In conclusion, we created Flc-LOM from an infectious cDNA clone of an attenuated CSFV vaccine strain and compared its biological properties with those of the parental LOM vaccine strain. Flc-LOM possessed all the biological and avirulent properties of its parental strain *in vitro* and *in vivo*. This clone will provide an opportunity for genetic manipulation of the LOM virus, including the creation of marker vaccines, and will be useful for identifying the genetic and serological determinants of CSFV virulence.

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References

1. **Aoki H, Sakoda Y, Nakamura S, Suzuki S, Fukusho A.** Cytopathogenicity of classical swine fever viruses that do not show the exaltation of Newcastle disease virus is associated with accumulation of NS3 in serum-free cultured cell lines. J Vet Med Sci 2004, **66**, 161-167.
2. **Cha SH, Choi EJ, Park JH, Yoon SR, Kwon JH, Yoon KJ, Song JY.** Phylogenetic characterization of classical swine fever viruses isolated in Korea between 1988 and 2003. Virus Res 2007, **126**, 256-261.
3. **Choi CH, Lee OS, Kim YH, An SH, Hwang EK.** Studies on the development of hog cholera live vaccine 1. cloning and identification of a new attenuated hog cholera virus. Res Rept Rural Dev Admin 1988, **30**, 42-48.
4. **de Smit AJ, Bouma A, van Gennip HGP, de Kluijver EP, Moormann RJM.** Chimeric (marker) C-strain viruses induce clinical protection against virulent classical swine fever virus (CSFV) and reduce transmission of CSFV between vaccinated pigs. Vaccine 2001, **19**, 1467-1476.
5. **de Smit AJ, van Gennip HGP, Miedema GKW, van Rijn PA, Terpstra C, Moormann RJM.** Recombinant classical swine fever (CSF) viruses derived from the Chinese vaccine strain (C-strain) of CSF virus retain their avirulent and immunogenic characteristics. Vaccine 2000, **18**, 2351-2358.
6. **De Vos CJ, Saatkamp HW, Huirne RB.** Cost-effectiveness of measures to prevent classical swine fever introduction into The Netherlands. Prev Vet Med 2005, **70**, 235-256.
7. **Dong XN, Chen YH.** Marker vaccine strategies and candidate CSFV marker vaccines. Vaccine 2007, **25**, 205-230.
8. **Drew T.** Classical swine fever (hog cholera). In: Office International des Epizooties (OIE) (ed.). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: Mammals, Birds and Bees. 6th ed. pp. 1092-1106, OIE, Paris, 2008.
9. **Genghini R, Tiranti I, Wittouck P.** Pig chromosome aberrations after vaccination against classical swine fever in field trials. Vaccine 2002, **20**, 2873-2877.
10. **Haegeman A, Dewulf J, Vrancken R, Tignon M, Ribbens S, Koenen F.** Characterisation of the discrepancy between PCR and virus isolation in relation to classical swine fever virus detection. J Virol Methods 2006, **136**, 44-50.
11. **Kang BJ, Kwon HJ, Kim SJ, Mun JB.** A field application of tissue culture attenuated hog cholera (LOM) vaccine. Res Rept Rural Dev Admin 1971, **17**, 27-33.
12. **Kang BJ.** Studies on low virulent hog cholera virus (LOM-E+) . Doctoral thesis, Azabu University Japan 1971.
13. **Kim B, Song JY, Tark DS, Lim SI, Choi EJ, Kim J, Park CK, Lee BY, Wee SH, Bae YC, Lee OS, Kwon JH, Kang WC, Kim TY, Kim JH, Lee JH, Kang MI.** Feed contaminated with classical swine fever vaccine virus (LOM strain) can induce antibodies to the virus in pigs. Vet Rec 2008, **162**, 12-17.
14. **Kumagai T, Shimizu T, Matumoto M.** Detection of hog cholera virus by its effect on Newcastle disease virus in swine tissue culture. Science 1958, **128**, 366.
15. **Lee C, Calvert JG, Welch SKW, Yoo D.** A DNA-launched reverse genetics system for porcine reproductive and respiratory syndrome virus reveals that homodimerization of the nucleocapsid protein is essential for virus infectivity. Virology 2005, **331**, 47-62.
16. **Mayer D, Thayer TM, Hofmann MA, Tratschin JD.** Establishment and characterisation of two cDNA-derived strains of classical swine fever virus, one highly virulent and one avirulent. Virus Res 2003, **98**, 105-116.
17. **Meyers G, Thiel HJ, Rümenapf T.** Molecular cloning and nucleotide sequence of the genome of hog cholera virus. Virology 1989, **171**, 555-567.
18. **Meyers G, Rümenapf T.** Classical swine fever virus: recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic defective interfering particles. J Virol 1996, **70**, 1588-1595.
19. **Moormann RJM, van Gennip HGP, Miedema GKW, Hulst MM, van Rijn PA.** Infectious RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. J Virol 1996, **70**, 763-770.
20. **Nishimura Y, Sato U, Hanaki T, Nobuto K.** Studies on the tissue culture of hog cholera virus. II. Neutralization test by means of the influence of hog cholera virus infection on newcastle disease virus infection (HEIC method). Nihon Juigaku Zasshi 1964, **26**, 133-140.
21. **Risatti GR, Borca MV, Kutish GF, Lu Z, Holinka LG, French RA, Tulman ER, Rock DL.** The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. J Virol 2005, **79**, 3787-3796.
22. **Ruggli N, Tratschin JD, Mittelholzer C, Hofmann MA.** Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. J Virol 1996, **70**, 3478-3487.
23. **Rümenapf T, Unger G, Strauss JH, Thiel HJ.** Processing of the envelope glycoproteins of pestiviruses. J Virol 1993, **67**, 3288-3294.
24. **Sakoda Y, Ozawa S, Damrongwatanapokin S, Sato M, Ishikawa K, Fukusho A.** Genetic heterogeneity of porcine and ruminant pestiviruses mainly isolated in Japan. Vet Microbiol 1999, **65**, 75-86.
25. **Sambrook J, Russell DW.** Molecular Cloning. 3rd ed. pp. 1.31-1.162, Cold Spring Harbor Laboratory Press, New York, 2001.
26. **Sato U, Hanaki T, Nobuto K.** Attenuation of the hog cholera virus by continuous cell-virus propagation. 3. Growth interference of Newcastle disease virus by attenuated hog cholera virus and its application to virus titration and the neutralization test. Arch Gesamte Virusforsch 1969, **26**, 1-10.
27. **van Gennip HGP, Bouma A, van Rijn PA, Widjojo-atmodjo MN, Moormann RJM.** Experimental non-transmissible marker vaccines for classical swine fever (CSF) by trans-complementation of E^{rns} or E2 of CSFV. Vaccine 2002, **20**, 1544-1556.
28. **van Oirschot JT.** Vaccinology of classical swine fever: from lab to field. Vet Microbiol 2003, **96**, 367-384.
29. **van Regenmortel MHV, Fauquet CM, Bishop DHL, Carsten EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB.** Virus

Taxonomy. 1st ed. pp. 867-872, Academic Press, San Diego, 2000.

30. **Vassilev VB, Collett MS, Donis RO.** Authentic and chimeric full-length genomic cDNA clones of bovine viral diarrhea virus that yield infectious transcripts. J Virol 1997, **71**, 471-478.
31. **Wee SH, Park CK, Jeong JM, Kim CH, Hwang IJ, Kim SJ, Yoon H, Lee ES, Nam HM, Park JY, Moon OK.** Outbreaks of classical swine fever in the Republic of Korea in 2003. Vet Rec 2005, **157**, 113-115.
32. **Widjojoatmodjo MN, van Gennip HGP, Bouma A, van Rijn PA, Moormann RJM.** Classical swine fever virus E^{rns} deletion mutants: *trans*-complementation and potential use as nontransmissible, modified, live-attenuated marker vaccines. J Virol 2000, **74**, 2973-2980.
33. **Xu J, Mendez E, Caron PR, Lin C, Murcko MA, Collett MS, Rice CM.** Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. J Virol 1997, **71**, 5312-5322.