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Infective viruses produced from full-length complementary DNA of swine vesicular disease viruses HK/70 strain

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Abstract The full-length cDNA clone of swine vesicular disease virus HK/70 strain named $pSVOK_{12}$ was constructed in order to study the antigenicity, replication, maturation and pathogenicity of swine vesicular disease virus. *In vitro* transcription RNA from $pSVOK_{12}$ transfected IBRS-2 cells and the recovered virus RNA were isolated and sequenced, then indirect hemagglutination test, indirect immunofluorescence assays, eleectron microscope test, 50% tissue culture infecting dose ($TCID_{50}$) assays and mouse virulence studies were performed to study the antigenicity and virulence of the recovered virus. The result showed that the infectious clones we obtained and the virus derived from $pSVOK_{12}$ had the same biological properties as the parental strain HK/70. The full-length infectious cDNA clone, $pSVOK_{12}$, will be very useful in studies of the antigenicity, virulence, pathogenesis, maturation and replication of SVDV.

Keywords: swine vesicular disease virus, full-length cDNA, infectious clone, $pSVOK_{12}$, reverse genetics.

Swine vesicular disease (SVD) is a highly contagious viral disease of pigs. Symptoms are clinically indistinguishable from those caused by other vesicular disease viruses, such as foot and mouth disease (FMD) virus, vesicular stomatitis (VS) virus and vesiviruses (which include vesicular exanthema of swine virus (VESV)), so SVD is classified as a list A disease by the Office International des Epizooties (OIE)[1]. The causative agent, swine vesicular disease virus (SVDV), is a member of the genus *Enterovirus* within the family *Picornaviridae*. It is a non-enveloped virus, containing a single-strand RNA genome of positive sense which is approximately 7.4 kb nucleotides in length[2,3] with a poly (A) tail at the 3′ end, and can act directly as messenger RNA in host cells. SVDV is both antigenically and genetically closely related to the human pathogen coxsackievirus B5 (CV-B5)[4−7], although pigs inoculated with CV-B5 do not show overt clinical signs of SVD[8]. It is possible that SVDV have potential harm to the human health. For example, Severe Acute Respiratory Syndrome (SARS) outbroke in 2002－2003 in southern China. It has been already reported that species of bats are natural host of coronavirus closely related to those responsible for SARS outbreak[9]. We also should attach importance to the questions of interspecific barrier and origin of the etiological agent, SVDV (or CV-B5), to prevent and control future outbreaks of SVDV or CV-B5.

SVD was first observed in Italy in 1966[10]. Since then, outbreaks have been reported in Europe and in the Far East. Although SVDV was largely eliminated from Europe during the 1970s－1980s, a new strain of SVDV, possibly originating from the Far East, prevailed in Europe during 1992. Subsequent report came from the Netherlands, Italy, Spain, Portugal and Belgium. In 2003/4, clinical outbreaks of SVD were only reported in Portugal, while subclinical infection was continuously detected in Southern Italy[1]. Outbreaks continue to occur in Italy and the disease is thought still to be present in China including Hong Kong region, and possibly other countries in Asia[11]. Once introduced, SVD can be a disease difficult to eradicate, and improved methods of control would be highly beneficial.

The production of infectious cDNA copies of viral genomes now allows precise study of the determinants of virulence at the molecular level. Full-length infectious cDNA constructs of the RNA genome of poliovirus were first described by Racaniello and Baltimore[12]. From then on, several infectious cDNAs corresponding to the genomes of Picornaviruses have been constructed[11−15], which make it possible to apply the technique of *in vitro* mutagenesis, known as reverse genetics, to

the analysis of viral function and the development of recombinant vaccines. Poliovirus, like SVDV, a member of the genus enterovirus, has been successfully controlled using the live attenuated Sabin vaccines[16]. Analogically, it might therefore be possible to get a live attenuated vaccine for SVD. Three infectious cDNA clones of SVDV have been established, but these cDNA clones were based on H/3′76[17], J′73[11] and NET/1/92[18]. J1′73 and H/3′76 were isolated from Japan, and NET/1/92 was isolated from Netherlands. To date, there is no report available about infectious cDNA clones of some SVDV isolated from China. In order to understand the mechanisms of molecular pathology of SVDV and develop effective vaccines, we have achieved an infectious clone of SVDV HK/70 strain.

1 Materials and methods

1.1 Virus and cells

The parental SVDV strain HK/70 was isolated from Hong Kong during the 1970 outbreak. The virus was propagated on IBRS-2 cells in DMEM with 5% fetal bovine serum (FBS) and antibiotics. After having been incubated for 48 h at 37℃, more than 90% of the cells showed cytopathology. Cell cultures were harvested and frozen and thawed three times. Prior to use, the virus suspension was centrifugated at 6000 *g* for 10 min and stored at −70℃. IBRS-2 cells was cultured in Dulbecco's Modified Eagle's Minimal Medium (DMEM) with Earle's salt solution supplemented with 10% FBS.

1.2 Construction and sequencing of full-length cDNA clone

By RACE, two overlaping cDNA fragments (5′ PCR and 3′ PCR fragment) covering the full genome of swine vesicular disease virus strain HK/70 were amplified from total RNA extracted from infected IBRS-2. 5′ PCR fragment restricted was cloned into pOK_{12} vector as recombinant plasmid $p5'OK_{12}$ by using enzyme of *Aat* II and *Xho* I, and the 3′ PCR restricted fragment was cloned into $p5'OK_{12}$ vector as recombinant plasmid $pSVOK_{12}$ by using enzyme of *Xba* I and *Bss*H II. The recombinant plasmid $pSVOK_{12}$ encoding full-length cDNA of SVDV HK/70 strain would be sequenced and analyzed.

1.3 In vitro transcription

The single-digested plasmid $pSVOk_{12}$ with *Psp*1406 I and two-digested $pSVOk_{12}$ with both *Psp*1406 I and *Aat* II were treated with proteinase K and purified by phenol/chloroform extraction and ethanol precipitation, respectively. The sediment was dissolved in 20 μL RNase free water. The sample was used for *in vitro* transcription with the transcription kit (Stratagene) according to the manufacurer's instructions. The RNA was purified by acid phenol-chloroform, followed by isopropanol precipitation and redissolved in Tris-EDTA buffer by heating at 70℃. To check the size and quality of the *in vitro*-transcribed RNA, a sample was denatured in urea-based RNA sample buffer (New England Biolabs) and electrophoresed on a 1% native agarose gel in Tris-borate-EDTA buffer with 1 μg ethidium bromide per mL.

1.4 Transfection of IBRS-2 cells with RNA

IBRS-2 cells were seeded in six-well plates (200000 cells/well in 2 mL of medium) and grown overnight to approximately 70%—80% confluency. Then 5 μg *in vitro*-transcribed RNA was mixed with 10 μL DMRIE-C (1,2-dimyristyloxypropy l-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol) (Invitrogen) and added directly to wells. Transfection of the mixture was performed in IBRS-2 with lipofectin according the manufacturer's protocol (Invitrogen). DMRIE-C without RNA was added to IBRS-2 cells as a negative control. IBRS-2 cells were transfected with viral RNA from the parental HK/70 isolate as a positive control. This control RNA was extracted from the viral suspension with RNeasy (Qiagen). After 4 h of exposure to DMRIE-C and RNA, the monolayers were washed, and fresh medium was added. The incubation continued up to 48 h. To increase virus titers, the transfected cells cultures was propagated on IBRS-2 cells for six times.

1.5 RNA isolation and sequencing analysis

Total RNA was isolated from cell culture using an RNA easy mini kit (Qiagen), RT-PCR was performed using SVDV-specfic primers (Table 1) and PCR products was sequenced by TaKaRa Biotechnology (Dalian) Co., Ltd. The primer was designed according to the published sequence of SVDV(ID (identification code for sequence in GenBank): AY429470).

1.6 Reversed indirect hemagglutination test

Approximately 1 mL IBRS-2 cells were transfected with synthetic virus. The cells were collected and incu-

Table 1 Primers used for constructing and sequencing the full-length genome of SVDV

Primer	Nucleotide sequence	Position
SV5′up	5′-GTTGGAtCCAAGCTTTAATACGACTCACTATAGTTAAAACAGCCTGTGG-3′	1—25
R1	5′-GTCTCATGAGCACCGGTCTTTTGT-3′	787—764
SV5′low	5′-TGTTCTCGAGCGGCCGCTACCTGCAGGCGCCAGTGGTTTTCATGGTTG-3′	3218—3195
SV3′up	5′-GAATTCCTGCAGGAACGTTAATTAAAACCACTGGCGCCTTCGGGCAGCAG-3′	3160—3183
F1	5′-GCATATGGGGATGTGATAGATAGCTTC-3′	6888—6913
SV3′low	5′-TCCTCTAGAACGTT(T)$_{38}$-3′	3′-end

bated at 58℃ for 40 min, and then centrifugated at 800 *g* for 10 min. The suspension was stored. The suspension was diluted with two-fold serial dilution and added to 96 well flat-bottomed plates. Then 25 μL blood corpuscle and the antibody against SVDV were also added to the plates. The mixtures was stired for 2—3 min and then incubated at 20—35℃ for 2 h. If the phenomena of hemagglutination appeared in the wells, the suspension contained SVDV.

1.7 *Indirect immunofluorescence assays (IFA)*

IFA were used to detect viral protein expression in SVDV RNA-transfected IBRS-2 cells. After transfection, approximately 10^5 transfected cells were spotted onto 10-mm thick glass coverslips. Cells on coverslips were analyzed by IFA at various times posttransfection for viral protein synthesis. Cells were fixed in 3.7% paraformaldehyde with PBS, pH 7.5, at room temperature for 30 min, followed by incubation in –20℃ methanol for 30 min. The fixed cells were washed with PBS, incubated at room temperature for 45 min in SVDV immune mouse ascites fluid (1∶100 dilution), and further reacted with goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate at room temperature for 30 min (1∶100 dilution). The coverslips were washed with PBS, mounted to a slide using fluorescent mounting medium (KPL) and observed under a fluorescence microscope equipped with a video documentation system.

1.8 *Electron microscope*

5 μL sample was absorbed onto glow-discharged, carbon-coated copper grids. Grids were washed with water, stained with 2% uranyl acetate and air-dried. Specimens were examined with an H-7500 electron microscope (JEM-1200EX) at 80 kV.

1.9 *50% Tissue culture infecting dose (TCID$_{50}$) assay*

The virus suspension was diluted with 10^{-1}—10^{-9} serial dilutions, and the 50 μL dilution was added to ninety-six well flat-bottomed cell culture plates, every dilution being added into 8 wells. 100 μL IBRS-2 of a 10^6/ML dilution of the original suspension was added into the well which contained virus dilutions. Observations are made of the number of ensuing infected monolayers for each dilution 72 h after infection. The TCID$_{50}$ value was determined by the method of Reed and Muench[19].

1.10 *Mouse virulence studies*

Virus was diluted with 10-fold serial dilution in phosphate-buffered saline containing 1% calf serum. Two-day-old mice were injected intra-peritoneally with 0.2 mL the ten-fold dilution of the vorus. Infected animals were observed 72 h after infection. The LD$_{50}$ was determined as described by Read and Muench[19].

2 Results

2.1 *Sequencing results of full-length cDNA clone*

The sequencing results showed that the complete genome was 7401 nucleotides long (excluding the poly(A) tract) with a poly(A) tail at least 74 adenines, which can encode a single polyprotein of 2185 aa 5′ UTR (743 nt) and 3′ UTR (102 nt). T7 promoter was added at 5′ end of the full-length cDNA and *Psp*1406 I additional restriction sites were added at 3′ end of poly (A) tail.

2.2 *Analysis of the RNA transcript*

The RNA transcripts were analyzed on a formaldehyde-denaturing agarose gel. The results showed RNA fragments about 7.48 kb were obtained (Fig. 1). It is certain that the RNA transcripts were obtained from the full-length cDNA of HK/70.

2.3 *The results of transfection*

To obtain the recovered infectious virus from the full-length cDNA clone, IBRS-2 cells were transfected with the RNA in transfection reagent DMRIE-C. Apparent CPE was observed 24 h after incubation (Fig. 2).

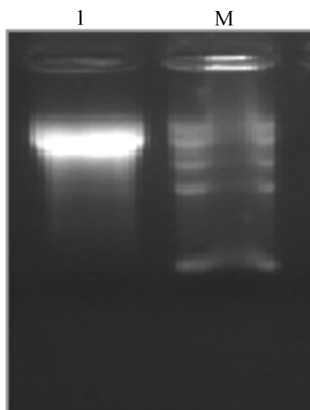

Fig. 1. SVDV RNA transcripts. Formaldehyde-denaturing 1.0% agarose electrophoresis of RNA transcripts. M, Molecular weight marker, 0.24－9.5 kb RNA Ladder (invitrogen).

This pathological effect was similar to that produced by wild virus, and the result in DNase and RNase treatments demonstrated that RNA rather than DNA was the infectious agent.

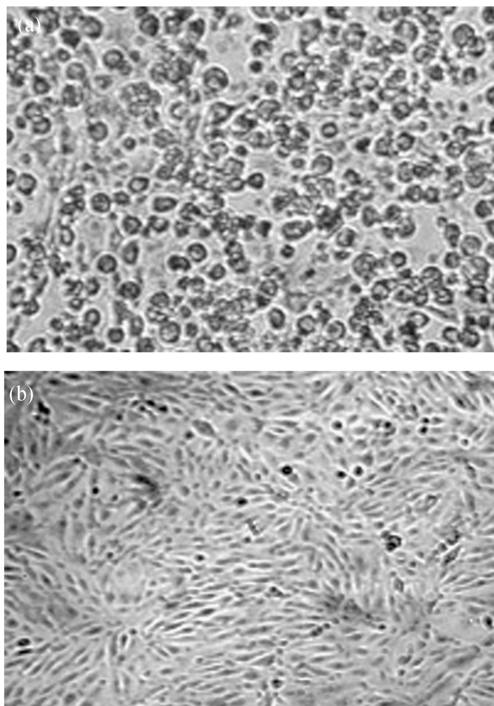

Fig. 2. CPE on IBRS-2 cell infected with the *in vitro* transcripts RNA. (a) IBRS-2 cells infected by transcripts; (b) normal IBRS-2 cells.

2.4 The results of RT-PCR and sequencing

The full-length genome of SVDV HK/70 was amplified by RT-PCR, as expected. Four DNA fragments were amplified (Fig. 3). And the results of sequencing also showed that the genome of the recovered virus was identical to that of the parent virus.

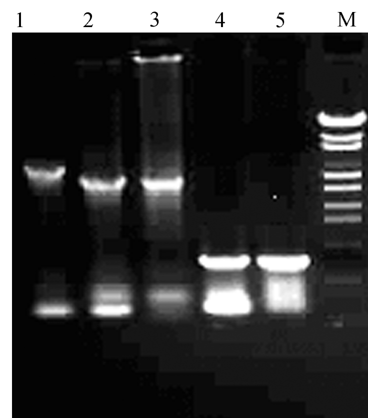

Fig. 3. Amplification of the genome of the recovered virus. 1, PCR products amplificated by SV3′up and SV3′low; 2 and 3, PCR products amplificated by SV5′up and SV5′low; 4, PCR products amplificated by SV5′up and R1; 5, PCR products amplificated by F1 and SV3′low; M, λ-*Eco*T14 I digest.

2.5 The results of reversed indirect hemagglutination test

To determine whether the cause of CPE was brought by SVDV or not, after four times passage, the cells transfected with RNA transcripts stably exhibited distinct CPE, and the transfected cells were analyzed by reverse indirect hemagglutination test.

The results showed that SVDV induced the CPE of cells. The retails (Fig. 4) were that some SVDVs recovered from RNA transcripts (Fig. 4(a) and (d)) from the *Psp*1406 I and *Aat* II-digested $pSVOK_{12}$ and no virus recovered from the transcripts (Fig. 4(b)) from *Psp*1406 I-linearized $pSVOK_{12}$. Fig. 4(c) shows a negative control group.

2.6 The results of IFA

To determine whether viral protein was expressed in SVDV RNA-transfected IBRS-2 cells, SVDV-positive IBRS-2 cells were analyzed by IFA. The normal cell line IBRS-2 was also analyzed by IFA as a negative control (Fig. 5(b)). The results showed that there was viral protein expression in the transfected cells (Fig. 5(a)).

2.7 Electron microscopy

The morphology of SVDV particles negative stained with phosphotungstate is shown in Fig. 6. Immune electron microscopic observation of the virus particles revealed that the particles were rotund with a diameter of 26 nm.

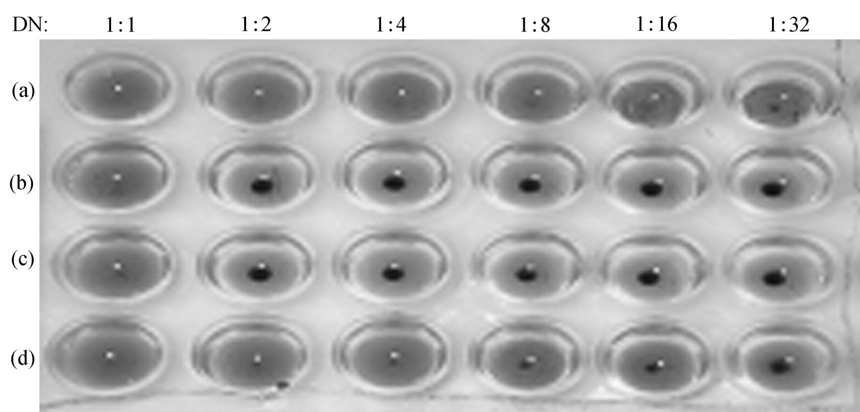

Fig. 4. SVDV detection of transfected cell IBRS-2 by reverse indirect hemagglutination test. DN, Dilution number of the suspension. The dilution number is shown at the top as from 1∶1 to 1∶32.

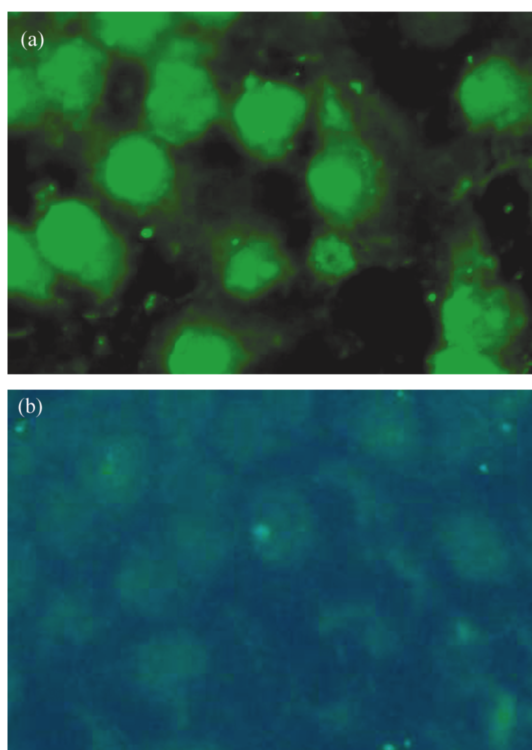

Fig. 5. The results of immunofluonrescence. (a) IBRS-2 cells infected by the virus from $pSVOK_{12}$; (b) normal IBRS-2 cells.

2.8 *The value of $TCID_{50}$*

To further compare growth characteristics of the parent virus and the virus derived from the transcripts, growth kinetics of these viruses were examined by determining the $TCID_{50}$. The value of $TCID_{50}$ of the virus derived from $pSVOK_{12}$ averaged $10^{-4.83}$ and the parent virus averaged $10^{-3.67}$. The recovered virus had weaker growth property than that of the parent virus.

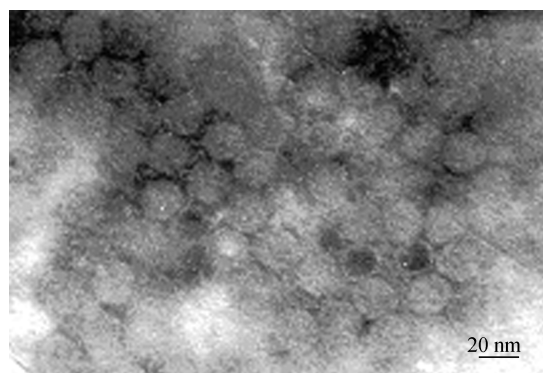

Fig. 6. Immune electron micrographs of negatively stained SVDV particles.

2.9 *Mouse virulence studies*

The virulence of the recovered viruses was evaluated with 2-d-old mice. The mice were intra-peritoneally injected with ten-fold dilution of the wild virus and the recombinant virus after four passages in IBRS-2 cells. Five mice were used per dosage and observed for 72 h. For the wild-type virus, the first mouse showed signs of hind leg paralysis 36 h after inoculation and died 16—24 h later. For the recombinant virus, the first mouse showed signs at 50 h and died 72 h after inoculation (Fig. 7). The LD_{50} of the recombinant virus was $10^{-7.5}$. In contrast, the LD_{50} of the wild virus was $10^{-7.625}$.

3 Discussion

The achievement of infectious clones (such as cDNAs or *in vitro*-transcribed RNA copies) corresponding to the genomes of RNA viruses has greatly enhanced the investigations. By the reverse genetics, we can understand the mechanisms of molecular pa-

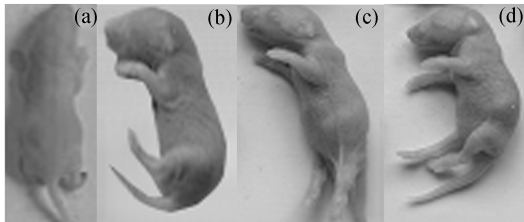

Fig. 7. Syndrome in mice caused by the virus derived from $pSVOK_{12}$. (a) Control mouse; (b) development of paralysis signs in hind legs of mouse inoculated with cloned virus; (c) and (d), the dead mouse inoculated with cloned virus after 72 h.

thology and immunology of the virus. Moreover, these clones can also be considered as "pool" to develop recombinant vaccines and other effective vaccines. Since the first infectious cDNA clone of poliovirus[12] was constructed, many cDNA copies from RNA genomes of members of the Picornaviridae have been obtained. For example, full-length cDNA copies constructed from two Japanese SVDV strains (J1′73 and H/3′76)[11,17] were used to study virulence differences between the two strains[20]. A full-length cDNA clone of the European SVDV NET/1/92 strain isolated in the 1990s[18] was also constructed. SVDV strains significantly differ from the Japanese strains at the genetic and antigenic level. Based on the monoclonal antibody (MAb) reaction pattern, SVDV NET/1/92 was classified into group IV together with all other SVDV strains isolated in Europe from 1992 to 1995[21]. The epitopes of newly prepared monoclonal antibodies were roughly mapped by fusion-PCR. Fine mapping of epitopes at the amino acid level was achieved by introducing single amino acid mutations in the genome of strain. Two new important amino acids in epitope formation were located in VP1; one was mapped in the C-terminal end and the other was thought to be located in the H-I loop[22].

In this work, the full-length cDNA clone of the HK/70 was constructed. This clone contained one nongenomic guanines at its 5′-end and an about 74 adenine poly(A) trail at its 3′-end, which is the longest up to now. The strain belongs to group II and showed similar pathogenicity to that of J1′73. The infectious clone would provide a convenient and precious tool for further researching SVDV.

But it is difficult to obtain an infectious clone, because several parameters may have influence on the infectivity of viral transcripts: (1) the heterogeneity of transcript population, (2) the presence of point mutation, and (3) the sequence at 5′ and 3′ ends (number and sequence of nonviral nucleotides, the presence of a cap structure at 5′ end or a poly(A) tail at 3′ end)[23].

A possible explanation to the heterogeneity of transcript population for the relatively low infectivity of most preparations might be that there is competition between incomplete nonreplicatable viral copies and full-length transcripts for interaction with viral and/or host factors involved in the replication process.

Because of the relatively poor fidelity of the RNA-and DNA-synthesizing enzyme involved in the production of transcripts, the point mutations and alteration in viral sequence are to be expected, which would decrease or abolish infectivity.

The nonviral nucleotides at the extremities of viral transcripts may have dramatic influence on the infectivity of viral transcripts[23,24]. As a general rule, 5′ extensions substantially decrease or abolish infectivity, whereas 3′ extension is more easily tolerated. The *Psp*1406 I-linearized $pSVOK_{12}$ contained 2 sequences of the T7 promoter on upstream of the full-length cDNA, so some transcripts with 5′ additional sequence could abolish the infectivity, as has been proved by our research. However, *in vitro* viral transcript with one or two additional guanine residues at the 5′-end is as infectious as virion RNA, and even could enhance the infectivity of transcripts[15].

The poly(A) tail may also play an important role in viral infectivity[25,26]. It has been reported that the poly(A) tract of FMDV RNA is required for the infectivity of FMDV, and the infectivity was found to increase with poly (A) length[27]. However, viral RNA from FMDV type A that contained less than 10 residues of adenosine at the 3′-end has been reported to have the same specific infectivity as viral RNA that contained approximately 40 residues of adenosine at the 3′-end[27]. Liu *et al.*[28] demonstrated that cDNA clones of FMDV with a poly (A) tail containing 16 A residues were infectious, which is consistent with the observations of other picornaviruses.

Classical biochemical approaches have identified long poly(C) tract as distinctive features of the genomes of cardioviruses and aphthoviruses. For FMDV, descriptive studies have suggested that changes in poly(C) tract length may be associated with changes in virulence[29]. So we should consider that the length of poly(C) tract has influence on the infectivity of the viruses while constructing a full-length cDNA clone.

The availability of an infectious cDNA clone of SVDV will allow us to address many questions of the

functions of viral gene products involved in infection and replication of this virus group. It will also allow us to study the antigenicity, virulence, pathogenesis, maturation and replication of SVDV. But the vitro-rescue methods have some limitations such as RNA degradation, costly reagents, complex program of manipulation and so on, and consequently it is difficult to get an infectious cDNA clone by the technique. To achieve successful infection, we must develop some methods with high efficiency for rescuing RNA virus, such as the methods for rescuing virus by *in vivo* transcription system.

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