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Short communication

Construction and characterization of a full-length infectious cDNA clone of foot-and-mouth disease virus strain O/JPN/2010 isolated in Japan in 2010

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Abstract

A full-length infectious cDNA clone of the genome of a foot-and-mouth disease virus isolated from the 2010 epidemic in Japan was constructed and designated pSVL-f02. Transfection of Cos-7 or IBRS-2 cells with this clone allowed the recovery of infectious virus. The recovered virus had the same in vitro characterization as the parental virus with regard to antigenicity in neutralization and indirect immunofluorescence tests, plaque size and one-step growth. Pigs were experimentally infected with the parental virus or the recombinant virus recovered from pSVL-f02 transfected cells. There were no significant differences in clinical signs or antibody responses between the two groups, and virus isolation and viral RNA detection from clinical samples were similar. Virus recovered from transfected cells therefore retained the in vitro characteristics and the in vivo pathogenicity of their parental strain. This cDNA clone should be a valuable tool to analyze determinants of pathogenicity and mechanisms of virus replication, and to develop genetically engineered vaccines against foot-and-mouth disease virus.

Keywords: Foot-and-mouth disease virus, full-length cDNA, Pathogenicity, O/JPN/2010 strain

Foot-and-mouth disease (FMD) is the most contagious disease of cloven-hoofed animals. Outbreaks of FMD cause enormous economic damage to the livestock industry by reducing livestock productivity and interrupting international trade of animals and their products. FMD virus (FMDV) is classified into the genus *Aphthovirus* within the family *Picornaviridae*. The genome of FMDV is composed of a single-stranded positive-sense RNA approximately 8.5 kb nucleotides in length with a poly (C) sequence at the 5' terminus of this genome, and a poly (A) sequence at the 3' terminus (Mason et al., 2003).

In Japan, FMD outbreaks occurred in 2000 and 2010; strains O/JPN/2000 and O/JPN/2010 were isolated from cattle during each outbreak, respectively (Sakamoto et al., 2002; Muroga et al., 2012). In the 2000 epidemic, cases were limited to four cattle farms and eradicated by slaughter of 740 cattle (Sugiura et al., 2001). Typical clinical signs were confirmed only in pigs following experimental infections with O/JPN/2000; inoculated Japanese Black cattle showed only mild clinical signs, and Holstein cattle and goats did not demonstrate clinical signs of infection, nor viral shedding (Yamakawa et al., 2002). In contrast, the FMD outbreak in 2010 spread to 292 farms and inoculated pigs, Holstein cattle and goats showed clinical signs and significant viral shedding following experimental infections with O/JPN/2010 (Fukai et al., 2011; Onozato et al., 2014). These

48 differences in infectivity and pathogenicity were suspected to contribute to the relative severity of
49 the 2010 outbreak compared to the one in 2000; however, the molecular mechanisms underlying the
50 pathogenicity of O/JPN/2000 and O/JPN/2010 are not well understood.

51 To date, infectious cDNA clones have been constructed for the purposes of understanding viral
52 replication and pathogenicity at the molecular level as well as for vaccine development (Zibert et al.,
53 1990; Rieder et al., 1993; Garcia-Arriaza et al., 2004; Liu et al., 2004; van Rensburg et al., 2004;
54 Hema et al., 2009; Xin et al., 2009; Rajasekhar et al., 2013) and recombinant and site-directed
55 mutants have been used for genomic studies (Falk et al., 1992; Piccone et al., 1995; Ellard et al.,
56 1999; Beard and Mason, 2000; Pacheco et al., 2003; Botner et al., 2011;Seago et al., 2013; Xin et al.,
57 2014). In the present study, a full-length infectious cDNA clone of O/JPN/2010 (pSVL-f02) was
58 constructed and the recovered virus was compared to its parental strain by in vitro characterization
59 and experimental infection of pigs.

60 The FMDV used in this experiment (O/JPN/2010 290-1E) was isolated from the epithelial tissue
61 of diseased cattle from a farm, which was confirmed as the 235th affected premises of 292 in total
62 during the 2010 epidemic in Japan. Viral RNA of O/JPN/2010 290-1E was extracted using a High
63 Pure Viral RNA Kit (Roche Diagnostics, Tokyo, Japan). First-strand cDNA synthesis was performed

64 using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics) and random hexamer
65 primers. Polymerase chain reaction (PCR) was performed using Platinum *Taq* Polymerase High
66 Fidelity (Life Technologies, Tokyo, Japan), using primers which were designed based on the
67 sequence of the O/JPN/2010 290-1E isolate (GenBank accession number LC036265) or other
68 reports (Sup. Table 1). The PCR fragments were inserted into the pGEM-T Easy cloning vector
69 (Promega, Tokyo, Japan). The full-length cDNA was assembled with five cDNA clones covering the
70 complete genome of O/JPN/2010 290-1E using appropriate restriction enzymes and cloned
71 downstream of the SV40 late promoter in the pSVL vector (formerly Pharmacia Biotech, WI, USA)
72 (Fig. 1). The 5N-2 clone had a poly $(C)_{18}$ tract and the 3R-L4 had a poly $(A)_{27}$ tail confirmed by
73 sequencing.
74 African green monkey kidney cell line Cos-7 and porcine kidney cell line IBRS-2 were grown
75 in Minimum Essential Medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) in a 12 well culture
76 plate. At 70–90% confluency, the cells were transfected with 0.1–1000 ng of pSVL-f02 using
77 Lipofectamine 3000 (Life Technologies) and incubated at 37°C, in 5% CO_2. In Cos-7 and IBRS-2
78 monolayers transfected with 1000 ng of pSVL-f02 DNA, distinct cytopathic effect (CPE) was
79 observed as soon as 1 day following transfection. At other doses, weak CPE were observed at 1 day

post-transfection in both cell types transfected with 100 ng of pSVL-f02, but not in cells transfected with 0.1-10 ng construct up to 3 days post-transfection. The supernatants and cells were collected at 3 days post-transfection, and virus was titrated in porcine kidney cell line, LFBK-alphavbeta6 (LaRocco et al., 2013); at least 100 ng and 10 ng of the plasmid were necessary to produce infectious virus in Cos-7 and IBRS-2 cells, respectively (Sup. Table 2A). After transfection using 1000 ng of pSVL-f02, supernatant or supernatant and cells were subjected to three freeze/thaw cycles, clarified and collected at 1 to 3 days post-transfection. The viral titers of these samples were determined as 50% tissue culture infectious dose ($TCID_{50}$) in LFBK-$\alpha_v\beta_6$ cells. The highest titers were detected at 2 and 1 day(s) post-transfection in Cos-7 and IBRS-2 cells, respectively (Sup. Table 2B).

The infectious virus from Cos-7 cells was passaged three times on the fetal goat tongue cell line, ZZR-127 (Brehm et al., 2009), then subsequently on baby hamster kidney cell line, BHK and ZZR-127 cells to obtain a high titer viral sample ($10^{7.3}$ $TCID_{50}$/0.1mL) for subsequent studies. The antigenicity of the recovered virus vSVL-f02 was examined by virus neutralization tests using sera collected from pigs inoculated with FMDV O/JPN/2010 290-1E isolate (Nos. 1 and 3) or the recovered virus vSVL-f02 (Nos. 5 and 6) were determined using each virus as an antigen (Sup. Table

96 3). There were no remarkable differences between the antibody titers. The antigenicity of the
97 vSVL-f02 was also examined by an indirect immunofluorescence test using FMDV-specific
98 monoclonal antibody (MAb) 1H5 which was raised against the O/JPN/2000 strain and reacts with all
99 seven serotypes of FMDV (Morioka et al., 2009) (Fig. 2). The MAb 1H5 reacted with the recovered
100 virus vSVL-f02. The plaques of the vSVL-f02 and the parental 290-1E isolate on LFBK and IBRS-2
101 cell monolayers were visualized by staining with crystal violet, and similar plaque growth was
102 confirmed (Fig. 3). In addition, vSVL-f02 and the parental 290-1E isolate were inoculated onto
103 LFBK cell monolayers cultured in 25-cm^2 flasks at a multiplicity of infection (MOI) of 2, then the
104 culture supernatant was harvested at subsequent time points and the amount of virus present was
105 determined (Fig. 4). Growth curves of both viruses reached a plateau at 12 hours post inoculation
106 and there were no significant differences in virus titers at each time points of the growth step
107 calculated by Student's t-test. Therefore, growth rates of these viruses were not significantly
108 different, and the in vitro characteristics of the recovered virus vSVL-f02 were demonstrated to be
109 identical to those of the parental strain 290-1E.
110 To test pathogenicity, the parental 290-1E isolate or recovered virus vSVL-f02 were inoculated
111 into pigs. Six 2-month-old pigs were intradermally inoculated with 0.1 ml of $10^{5.0}$ $TCID_{50}$ of the

viruses (group 1: 290-1E, group 2: vSVL-f02) at the right and front heel bulbs. Pigs were observed for the appearance of clinical signs daily until 14 days post inoculation (dpi). Sera, saliva, and nasal discharge were collected as described in our previous report (Fukai et al., 2015). Animal experiments were authorized by the Animal Care and Use Committee of the National Institute of Animal Health (NIAH) (authorization number: 14-060) and were performed in a high-containment facility at the NIAH. All pigs infected with either 290-1E isolate or vSVL-f02 developed vesicles by 1 or 2 dpi on their feet, lips, and tongues (Table 1). Viruses were isolated from the sera (1–3 dpi), saliva (1–6 dpi), and nasal swabs (2–3 dpi) of the 290-1E inoculated pigs, and from the sera (1–3 dpi), saliva (2–5 dpi) and nasal swabs (1–5 dpi) of the vSVL-f02 inoculated pigs by using LFBK cells. Viral genes were detected in the sera (1–3 dpi), saliva (1–14 dpi) and nasal swabs (2–10 dpi) collected from 290-1E inoculated pigs, and in the sera (1–8 dpi), saliva (2–14 dpi) and nasal swabs (1–9 dpi) collected from vSVL-f02 inoculated pigs by reverse transcription PCR (RT-PCR) using the FM8-9 primer set previously described (Sakamoto et al., 2002). Moreover, virus titers were determined by the microtitration method using LFBK cells and viral RNA loads were determined by real-time RT-PCR using primers and a probe described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013 of World Organization for Animal Health. Viral titers in the

128 sera, saliva, and nasal swabs collected from pigs in group 1 were $10^{2.1}$–$10^{6.1}$ $TCID_{50}$/ml, $10^{2.6}$–$10^{6.3}$

129 $TCID_{50}$/ml and $10^{2.6}$–$10^{6.6}$ $TCID_{50}$/ml, respectively, while the viral RNA loads were $10^{6.6}$–$10^{9.8}$

130 copies/ml, $10^{7.5}$–$10^{10.6}$ copies/ml and $10^{7.7}$–$10^{10.2}$ copies/ml (Sup. Tables 4 and 5). In group 2, viral

131 titers were $10^{2.8}$–$10^{5.3}$ $TCID_{50}$/ml, $10^{2.6}$–$10^{5.6}$ $TCID_{50}$/ml and $10^{2.6}$–$10^{6.9}$ $TCID_{50}$/ml, respectively,

132 while the viral RNA loads were $10^{6.9}$–$10^{9.5}$ copies/ml, $10^{7.7}$–$10^{10.1}$ copies/ml and $10^{7.4}$–$10^{10.3}$

133 copies/ml (Sup. Tables 4 and 5). Antibodies were detected by liquid-phase blocking enzyme-linked

134 immuno-sorbent assay (LPBE) (Biological Diagnostic Supplies Limited, Scotland, UK) from 5 or 6

135 dpi in both groups and the antibodies against non-structural protein (NSP) of FMDV were detected

136 using the PrioCHECK FMDV NS Antibody ELISA Kit (Life Technologies) from 7 or 8 dpi, and 6–8

137 dpi in groups 1 and 2, respectively. Therefore, no significant differences in clinical signs, virus

138 isolation and titers, viral RNA loads and antibody responses were observed between groups 1 and 2.

139 In the present study, plasmid pSVL-f02 containing a full-length cDNA of FMDV O/JPN/2010

140 290-1E isolate was constructed and the vSVL-f02 virus was recovered following transfection of

141 mammalian cells. The pSVL-f02, which is based on pSVL, harbors a full-length FMDV cDNA

142 within the SV40 VP1 translational unit and carries the SV40 origin of replication. Therefore, the

143 replication efficiency of this plasmid should theoretically be greatly increased in Cos-7 cells which

144 express the SV40 large T antigen and result in high levels of protein expression following DNA
145 transfection (Mellon et al., 1981). In this study, contrary to expectations, transfection efficiency did
146 not increase in Cos-7 cells compared to IBRS-2 cells. The reason for this is unknown; however, it
147 was probably due to the difference in susceptibility to infection with FMDV between Cos-7 and
148 IBRS-2 cells.

149 The in vitro characteristics of vSVL-f02, including its antigenicity in the neutralization test,
150 indirect immunofluorescence, and plaque size and one-step growth, were identical to those of the
151 parental 290-1E isolate (Fig 2-4, Sup. Table 3). In the animal experiments, pigs inoculated with
152 either the vSVL-f02 or O/JPN/2010 290-1E isolate developed vesicles on their feet, lips and tongues
153 beginning at 1 or 2 dpi. In addition, there were no significant differences between groups 1 and 2 in
154 the dynamics of viral loads in serum, saliva, and nasal swabs or in the development of anti-FMDV
155 and anti-NSP antibodies (Table 1, Sup. Tables 4 and 5). These data demonstrate that the in vitro and
156 in vivo characteristics of the virus recovered from pSVL-f02 transfected cells were consistent with
157 those of parental virus. Therefore, we concluded that a full-length cDNA of FMDV O/JPN/2010
158 290-1E was successfully cloned and constructed. This infectious cDNA is therefore a valuable tool
159 to analyze the molecular mechanisms of differences in pathogenicity of O/JPN/2000 and

O/JPN/2010 strains, as well as to conduct further studies to understand the mechanisms of virus replication and determinants of pathogenicity of FMDV, and to develop genetically engineered vaccines.

Declaration of conflicting interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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264
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Figure Legends

Fig. 1. Construction of full-length cDNA clone of the FMDV O/JPN/2010 290-1E isolate. Five cDNA clones comprising the genome of the 290-1E isolate were ligated to each other using appropriate restriction enzymes and inserted into the pSVL plasmid to create a full-length cDNA identified as pSVL-f02.

Fig. 2. Indirect immunofluorescence test of IBRS-2 cells infected with vSVL-f02. Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) antibody (Life Technologies) was used for detection. Coverslips were mounted using ProLong Gold Antifade Reagent with DAPI (Life Technologies). (a) vSVL-f02, (b) No infection.

Fig. 3. Comparison of plaque size of 290-1E isolate and vSVL-f02. (a) and (c) 290-1E isolate inoculated onto IBRS-2 or LFBK monolayer cells, respectively, (b) and (d) vSVL-f02 inoculated onto IBRS-2 or LFBK monolayer cells, respectively. The cultures were fixed 1 day after the inoculation and stained with crystal violet.

Fig. 4. One-step growth curves of 290-1E isolate and vSVL-f02. The LFBK cell monolayers were inoculated with each virus at a MOI of 2 and incubated at 37°C. Samples of supernatant were collected at the indicated times and viral infectivity was determined using a standard plaque assay.

Table 1. Isolation of viruses, detection of viral genes, and detection of antibodies in clinical samples obtained from infected pigs

Group No.	Pig No.	Virus	Clinical sample	Days post infection											
				0	1	2	3	4	5	6	7	8	9	10	13/14
1	1	290-1E	Serum	-/-[a]	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
			Saliva	-/-	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/+	-/-
			Nasal swab	-/-	-/-	+/+	+/+	-/+	-/+	-/+	-/-	-/-	-/-	-/+	-/-
			LPBE[b]	＜32	＜32	＜32	＜32	＜32	45	90	181	256	181	362	181
			NSP[c]	-	-	-	-	-	-	-	+	+	+	+	+
	2		Serum	-/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
			Saliva	-/-	-/-	+/+	+/+	+/+	-/+	+/+	-/+	-/+	-/+	-/-	-/+
			Nasal swab	-/-	-/-	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-
			LPBE	＜32	＜32	＜32	＜32	＜32	＜32	45	90	181	181	256	181
			NSP	-	-	-	-	-	-	-	+	+	+	+	+
	3		Serum	-/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
			Saliva	-/-	-/-	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/+	-/-
			Nasal swab	-/-	-/-	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/-	-/+	-/-
			LPBE	＜32	＜32	＜32	＜32	＜32	＜32	32	45	90	181	256	362
			NSP	-	-	-	-	-	-	-	-	+	+	+	+
2	4	vSVL-f02	Serum	-/-	+/+	+/+	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

	Saliva	-/-	-/-	+/+	+/+	-/+	-/+	-/+	-/-	-/+	-/+	-/-	-/-
	Nasal swab	-/-	-/-	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/-
	LPBE	＜32	＜32	＜32	＜32	＜32	＜32	45	90	90	90	90	181
	NSP	-	-	-	-	-	-	-	-	+	+	+	+
5	Serum	-/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	Saliva	-/-	-/-	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/-
	Nasal swab	-/-	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/-	-/-	-/-
	LPBE	＜32	＜32	＜32	＜32	＜32	32	45	90	181	181	181	256
	NSP	-	-	-	-	-	-	-	+	+	+	+	+
6	Serum	-/-	+/+	+/+	+/+	-/-	-/-	-/-	-/+	-/+	-/-	-/-	-/-
	Saliva	-/-	-/-	+/+	+/+	+/+	-/+	-/+	-/+	-/-	-/+	-/-	-/+
	Nasal swab	-/-	-/-	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-
	LPBE	＜32	＜32	＜32	＜32	＜32	64	181	362	362	256	181	256
	NSP	-	-	-	-	-	-	+	+	+	+	+	+

Boxes in the table indicate the day at which obvious vesicles appeared in each pig.

[a] Isolation of virus/detection of virus gene.

[b] Antibodies against foot-and-mouth disease virus (FMDV) were detected and titrated by liquid-phase blocking enzyme-linked immuno-sorbent assay (LPBE).

[c] Antibodies against non-structural protein (NSP) of FMDV were detected using the PrioCHECK FMDV NS Antibody ELISA Kit.

Fig. 1

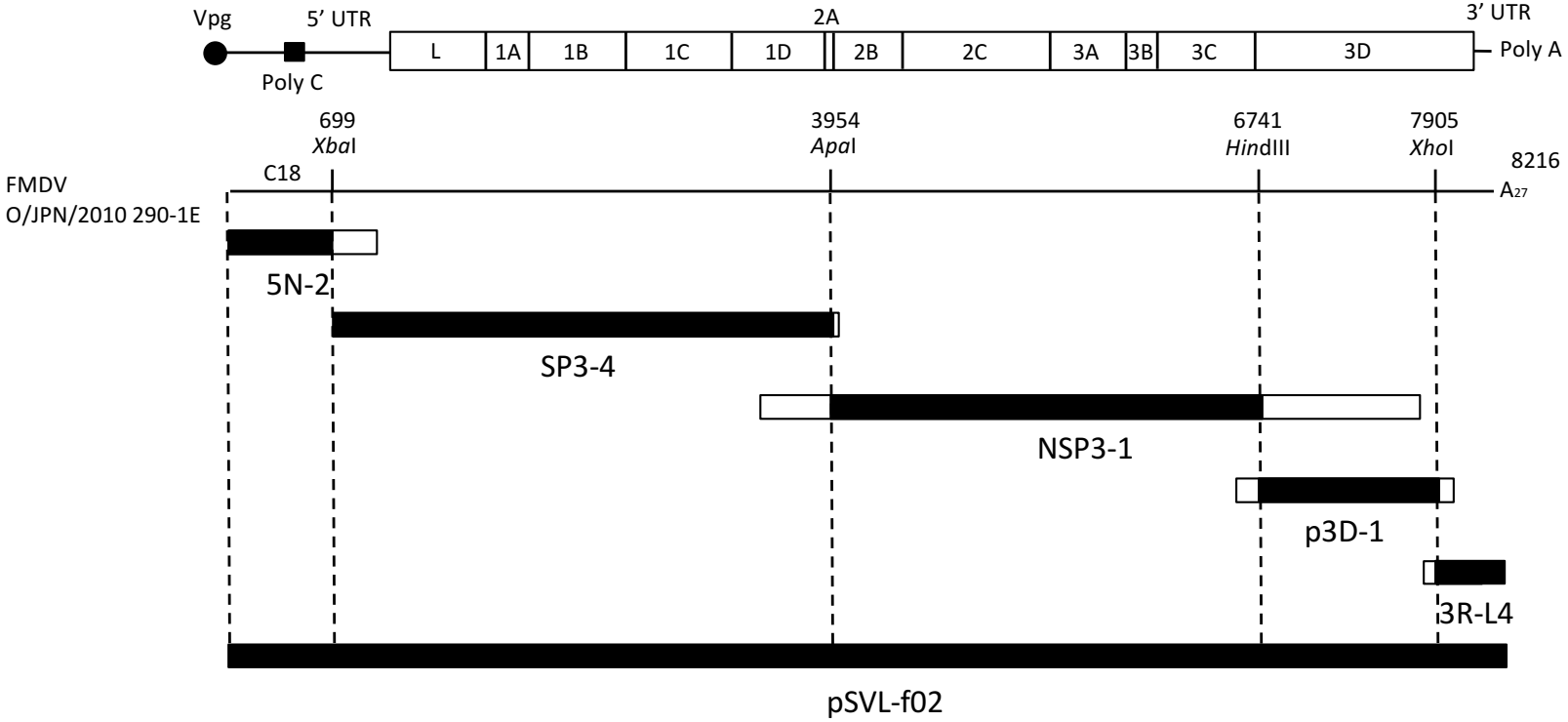

Vpg
5' UTR
2A
3' UTR
L
1A
1B
1C
1D
2B
2C
3A
3B
3C
3D
Poly A
Poly C
699
XbaI
3954
ApaI
6741
HindIII
7905
XhoI
8216
C18
FMDV
O/JPN/2010 290-1E
A27
5N-2
SP3-4
NSP3-1
p3D-1
3R-L4
pSVL-f02

Fig. 2

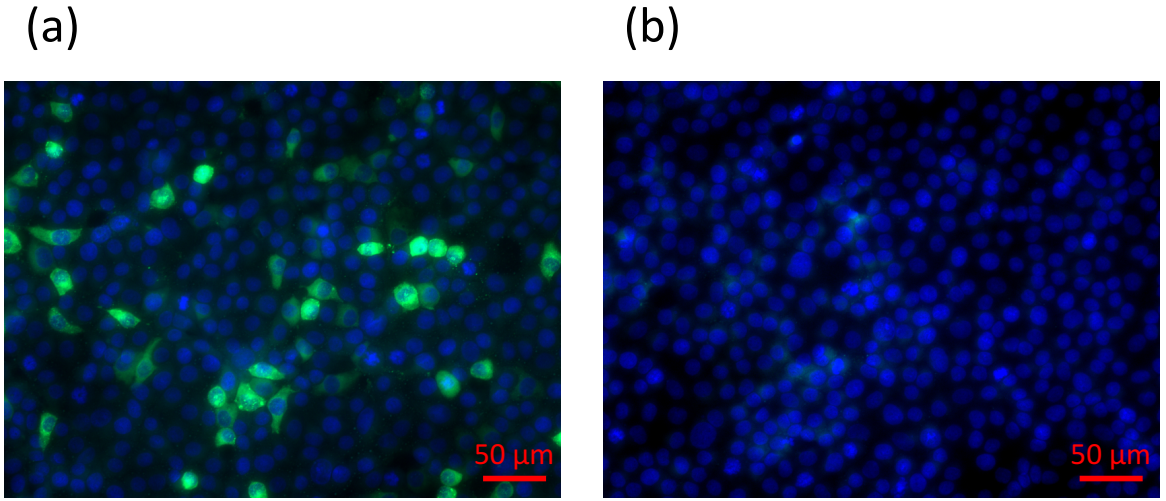


Fig. 3

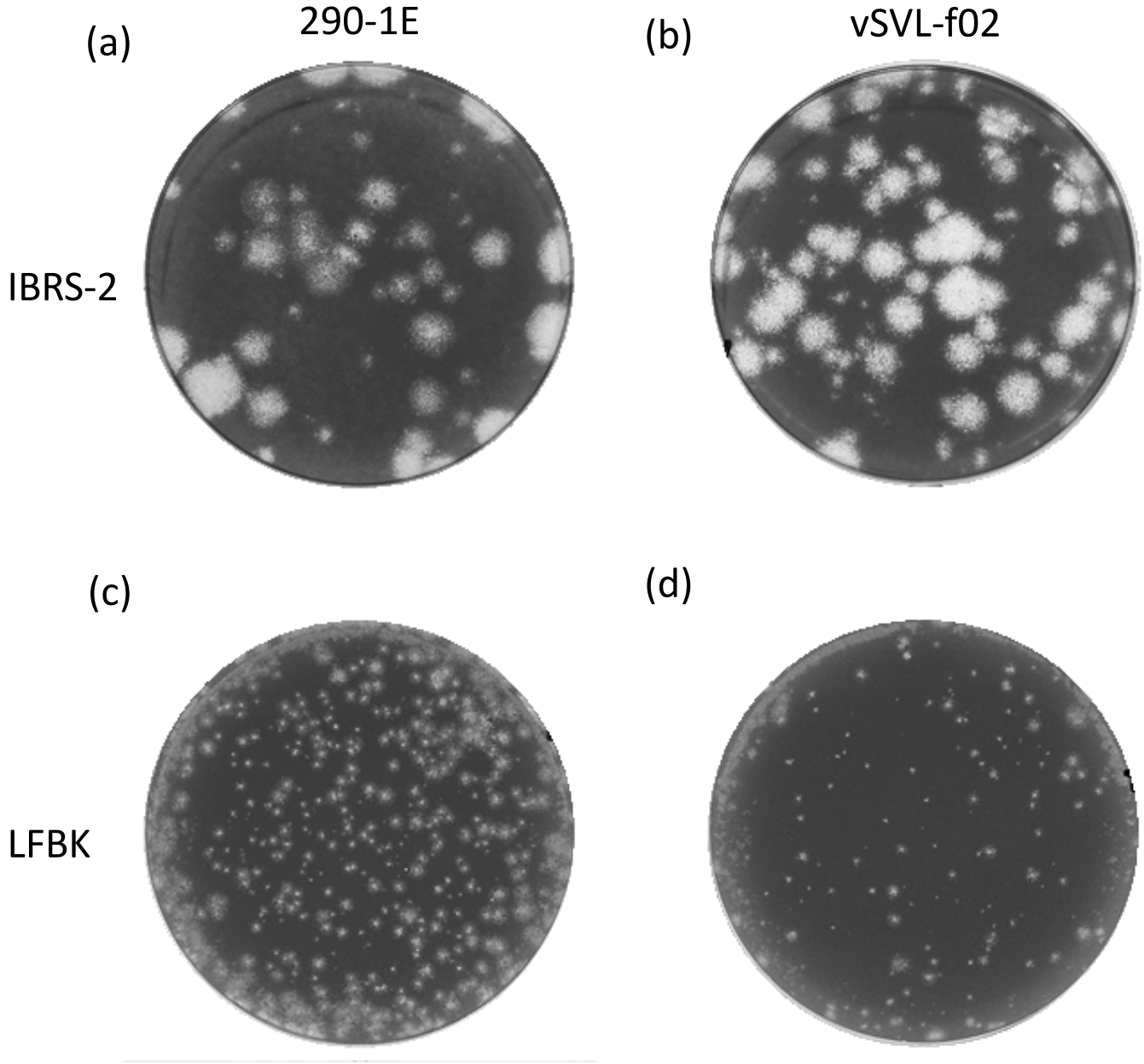
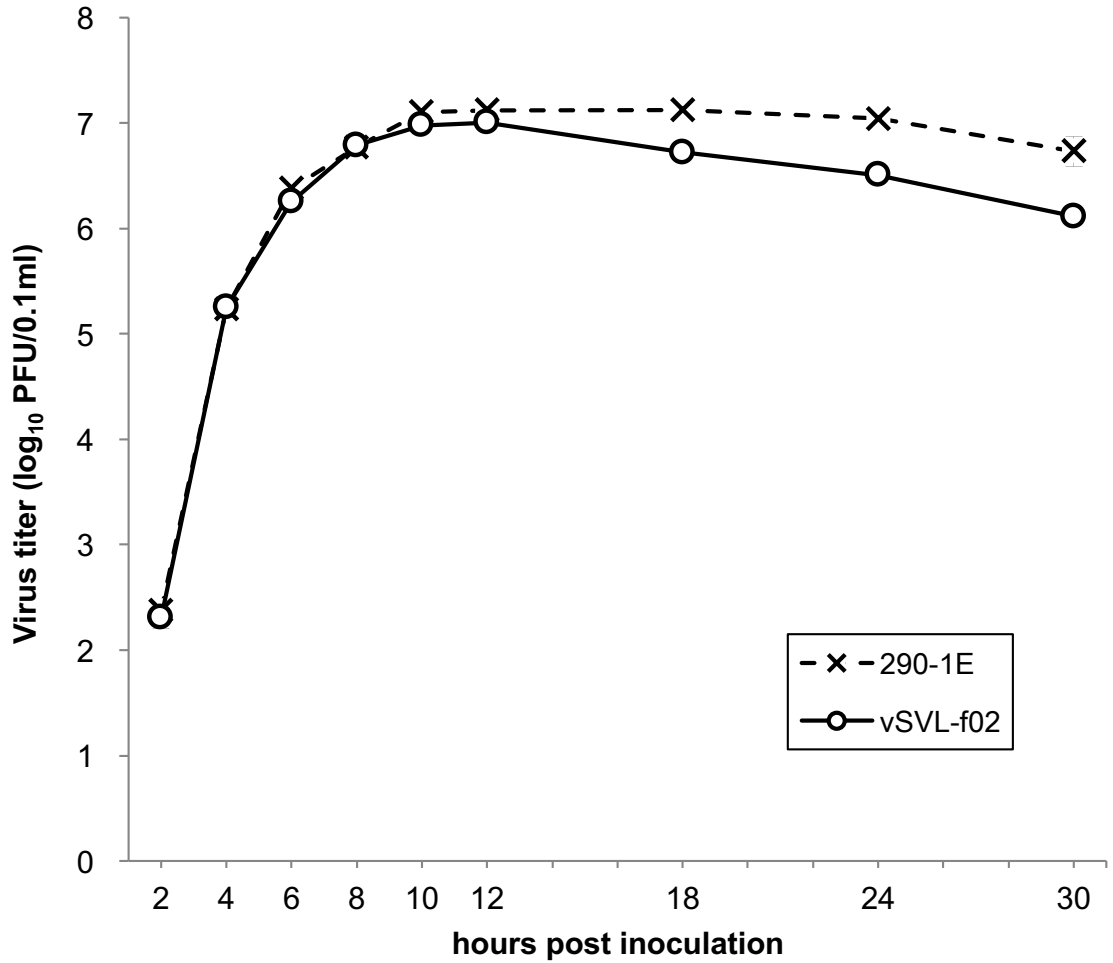


Fig. 4


Virus titer (log10 PFU/0.1ml)
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
18
24
30
hours post inoculation
290-1E
vSVL-f02