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# Studies on the molecular basis of the pathogenicity of foot-and-mouth disease virus

口蹄疫ウイルスの病原性の分子基盤に関する研究

Tatsuya Nishi

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# Abbreviations

| | |
|---|---|
| ASFV | African swine fever virus |
| BHK-21 cell | baby hamster kidney cell |
| BK cell | primary bovine kidney cell |
| BRAV | bovine rhinitis virus A |
| BRBV | bovine rhinitis virus B |
| Cos-7 cell | African green monkey kidney cell with CV-1 origin SV40 |
| CPE | cytopathic effect |
| CPK cell | cloned porcine kidney cell |
| CSFV | classical swine fever virus |
| DMEM | Dulbecco's modified Eagle's medium |
| dpi | days post-infection |
| dpc | days post-contact |
| ELISA | enzyme-linked immunosorbent assay |
| FMD | foot-and-mouth disease |
| FMDV | foot-and-mouth disease virus |
| G-H loop | loop connecting the βG and βH strands of protein |
| IB-RS-2 | *Instituto Biologico rim suino* and pig kidney number 2 |
| IRES | internal ribosome entry site |
| kb | kilobase |
| $LD_{50}$ | 50% lethal dose |
| LPBE | liquid-phase blocking ELISA |
| MCMC method | Markov chain Monte Carlo method |
| MDBK cell | Madin-Darby bovine kidney cell |

| | |
|---|---|
| MEM | Eagle’s minimum essential medium |
| ME-SA | Middle East-South Asia |
| ML method | maximum-likelihood method |
| MOI | multiplicity of infection |
| NIAH | National Institute of Animal Health |
| NSP | non-structural protein |
| OIE | World Organization for Animal Health |
| ORF | open reading frame |
| PFU | plaque forming unit |
| RGD | Arg-Gly-Asp |
| RT-PCR | reverse transcription polymerase chain reaction |
| rRT-PCR | real-time reverse transcription polymerase chain reaction |
| SAT | south African territories |
| SEA | South-East Asia |
| SVA | Senecavirus A |
| SVDV | swine vesicular disease virus |
| $TCID_{50}$ | 50% tissue culture infective dose |
| UTR | untranslated region |
| VNT | virus neutralization test |
| VP | viral protein |
| VSV | vesicular stomatitis virus |
| ZZ-R 127 cell | fetal goat tongue cell |

# Notes

Contents of the present thesis were published in the following articles.

1. **Nishi T, Onozato H, Ohashi S, Fukai K, Yamada M, Morioka K, Kanno T.** 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. Res Vet Sci 106, 165-169, 2016.

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2. **Nishi T, Yamada M, Fukai K, Shimada N, Morioka K, Yoshida K, Sakamoto K, Kanno T, Yamakawa M.** Genome variability of foot-and-mouth disease virus during the short period of the 2010 epidemic in Japan. Vet Microbiol 199, 62-67, 2017.

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3. **Nishi T, Kanno T, Shimada N, Morioka K, Yamakawa M, Fukai K.** Reverse transcription-PCR using a primer set targeting the 3D region detects foot-and-mouth disease virus with high sensitivity. Transbound Emerg Dis 66, 1776-1783, 2019.

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4. **Nishi T, Morioka K, Saito N, Yamakawa M, Kanno T, Fukai K.** Genetic determinants of virulence between two foot-and-mouth disease virus isolates which caused outbreaks of differing severity. mSphere 4, 4, 2019.

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# General Introduction

Foot-and-mouth disease (FMD) is the most contagious disease of mammals and causes severe economic damage to livestock industries. FMD virus (FMDV) is classified into the genus *Aphthovirus* of the family *Picornaviridae* (1). Its genome is composed of a single-stranded positive-sense RNA of approximately 8.4 kilobases (kbs) in length which is divided into an S-fragment and an L-fragment by a poly(C) sequence at the 5' untranslated region (UTR) of the genome (Fig. 1a). Translation of viral mRNA initiates at internal ribosomal entry site (IRES) by a cap-independent mechanism (2). The RNA is translated as a single long open reading frame (ORF) into a polyprotein. A series of post-translational proteolytic cleavages generate 12 proteins, L, viral protein (VP) 1-4, 2A-2C, and 3A-3D finally. The FMDV capsid surface is covered by VP1, 2 and 3, and held by VP4 which is buried within the virion (2). The loop connecting the βG and βH strands of protein (G-H loop) of VP1, including the Arg-Gly-Asp (RGD) amino acid sequence motif, binds to RGD-binding integrins as receptors of FMDVs in susceptible animals (3). To date, a total of five primary antigenic sites have been identified on the capsid (4, 5). Nonstructural proteins, 2A-2C and 3A-3D are involved in protein processing and genome replication (2). During the infectious cycle, 3C protein is responsible for most cleavages of the viral polyprotein. 3D protein is viral-encoded RNA-dependent RNA polymerase and catalyze

RNA replication. The replication cycle is finalized by the encapsidation of nascent positive-sense viral RNA and maturation cleavage of VP0 to VP2 and VP4.

FMDV isolates comprise the following immunologically distinct serotypes: O, A, C, Asia1, and South African Territories (SAT) 1–3 (Fig. 1b). Each serotype can be divided into genetically and geographically distinct topotypes based on comparisons of VP1 sequence (6). FMDV serotypes O and A have the broadest distribution, occurring in many parts of Africa, Asia, and Southern America. In Asia, regular outbreaks of FMD have been reported in India and mainland Southeast Asia (7). Sporadic introductions of viruses into FMD-free countries may cause outbreaks with devastating damages. During 1999-2002, the virus of serotype O Middle East-South Asia (ME-SA) topotype, PanAsia lineage caused widespread outbreaks in east Asia including China, Russia, Mongolia, South Korea, and Japan prior to those in South Africa and Europe (7). In 2010 and 2011, incursions of the FMDV serotype O, South-East Asia (SEA) topotype, Mya-98 lineage, normally restricted to countries in mainland Southeast Asia, caused extensive outbreaks across East Asian countries (8).

Domesticated cloven-hoofed animals including cattle, swine, sheep and goat, as well as wild animals including deer and wild boar are susceptible to FMD. FMDV-infected animals show vesicles on the mouth, nostrils, and around the breasts and feet as typical lesions. The pathogenicity and infectivity of FMDV in cattle or swine is strain-dependent, for example,

FMDV with atypical pathogenicity which showed high morbidity and mortality in swine but did not affect cattle was confirmed, and shown to have caused a devastating outbreak in Taiwan (9). In contrast, FMDVs which showed limited pathogenicity in cattle were isolated in South Korea and Argentina (10, 11). Several previous studies have reported that pathogenicity in hosts and virus growth in cell culture were related, and that the factors responsible were two substitutions on the 133rd amino acid in VP2 and the 56th amino acid in VP3, a deletion of $L^{pro}$, the part of a deletion in 3A, and IRES-3'UTR modulation (12-16). To date, however, few studies have described the genes responsible for FMDV pathogenicity among multiple topotypes.

Japan has experienced two FMD outbreaks over the last 100 years. These outbreaks were notably differed in severity. The 2000 outbreak was limited to four cattle farms, whereas the 2010 outbreak spread to 292 farms and resulted in the slaughter of about three hundred thousand animals (17, 18). In 2000, infected cattle showed only fever and salivation, and the typical clinical sign including vesicular development was not confirmed. In 2010, in contrast, the typical clinical signs of fever, salivation, ulcers in the mouth and vesicular development were all confirmed. While this different pathogenicity in the field might be one factor caused the difference in the severity of the two outbreaks, the molecular mechanisms underlying the pathogenicity of the virus are not well understood.

Thus, in the present thesis, the author investigated the molecular basis on the pathogenicity

of FMDV isolated in Japan in 2010. The present thesis consists of four chapters; in Chapter I, highly sensitive RT-PCR assay targeting a conserved region of the 3D domain of FMDV was established to enhance sensitivity of nucleic acid analyses for the diagnosis of FMD. In Chapter II, whole genome sequences of virus isolates obtained from each case of the 2010 epidemic in Japan were determined with the aim of elucidating their genome variability during a short period of an epidemic. In Chapter III, a full-length infectious cDNA clone of FMDV isolated in Japan in 2010 was constructed, and the recovered virus was compared to its parental strain by *in vitro* and *in vivo* characterization. In Chapter IV, the infectivity of two virus strains isolated in Japan in 2000 and 2010, which caused outbreaks of differing severity were compared in cell culture, suckling mice, and cattle. In addition, the genes responsible for the difference in the infectivity were evaluated using genetic recombinants between the two strains.

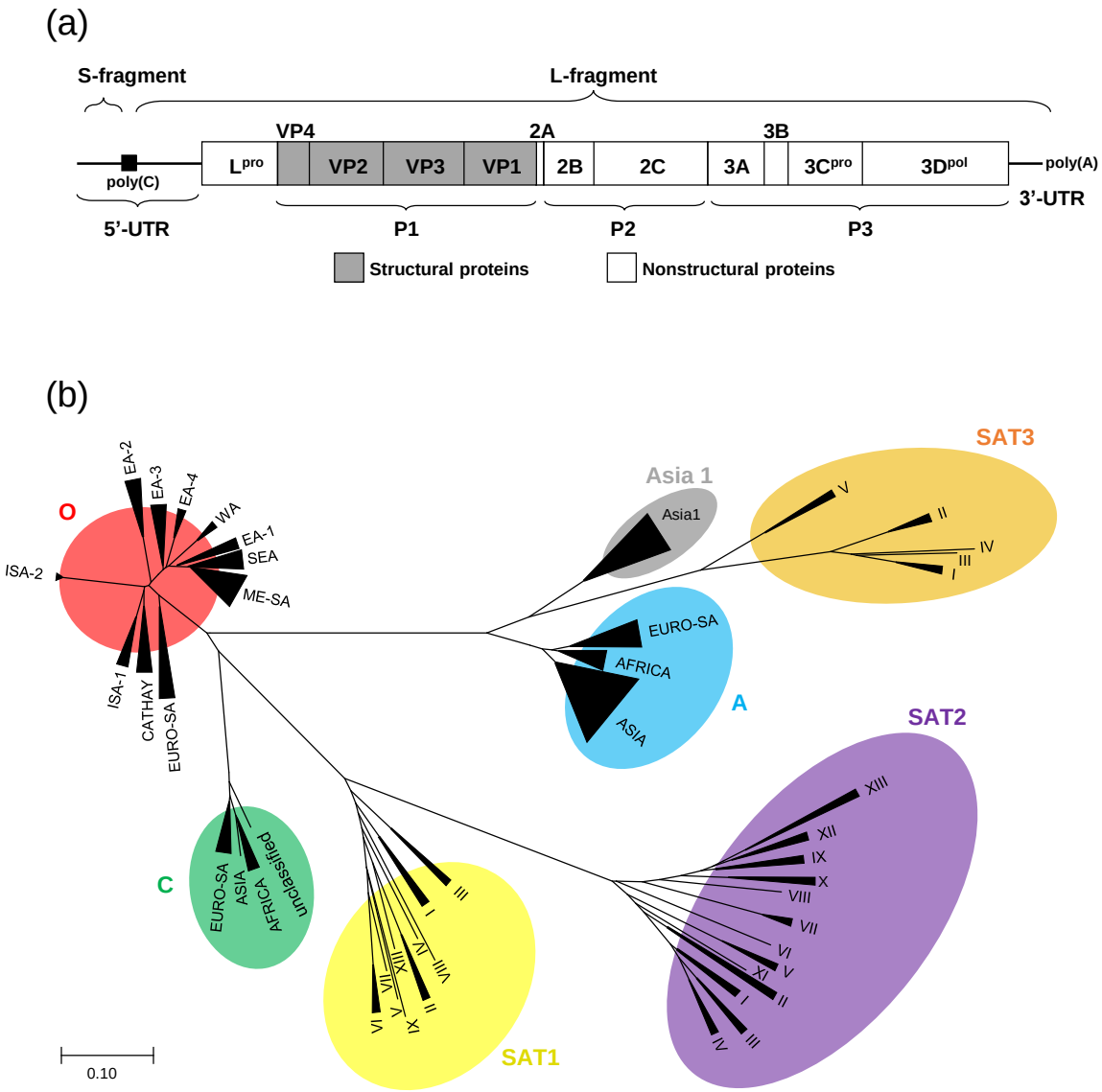


**Fig. 1. Schematic map of the FMDV genome and unrooted neighbour-joining tree showing genetic diversity of FMDV of the seven serotypes.**

(a) The viral proteins are shown in the boxed area. The structural and nonstructural proteins are indicated in gray and white boxes, respectively. pro, protease. pol, polymerase. (b) Full length of VP1 nucleotide sequences of FMDVs were used for phylogenetic analysis based on the Kimura 2-parameter model. Representative sequences for each FMDV serotype and topotype were obtained from the DDBJ/EMBL/GenBank databases according to protocols of VP1 sequencing for FMDV prototype (6).

# Chapter I

## Reverse transcription-PCR using a primer set targeting the 3D region detects foot-and-mouth disease virus with high sensitivity

# Introduction

FMD causes the most contagious disease affecting cloven-hoofed animals, and the control of FMD depends on early detection of infected animals. Therefore, rapid and accurate methods for diagnosing FMD are essential. FMDV is detected using cell culture techniques, enzyme-linked immunosorbent assays (ELISA), and nucleic acid analyses such as those described in the World Organization for Animal Health (OIE) Terrestrial Manual (19). Among the latter methods, reverse transcription polymerase chain reaction (RT-PCR) assays and real-time RT-PCR (rRT-PCR) assays rapidly detect FMDV with high sensitivity (20-22). Given that the FMDV genome mutates at a high rate, similar to that of other RNA viruses (23), a highly conserved region should be targeted for the detection of the seven serotypes by RT-PCR. Although these assays rapidly detect the FMDV, agarose gel-based RT-PCR assays may have the possibility of cross-contamination with PCR amplicons containing FMDV genes, and rRT-PCR tends to generate nonspecific signals, particularly from samples containing low RNA copy numbers. Therefore, PCR-based methods should be used together with other methods to diagnose FMD to avoid misidentification (24).

The OIE manual specifies a primer set for agarose gel-based RT-PCR assays (named 1F/R) that targets the 5'-UTR (25) and primer sets for rRT-PCR assays that target the 5'-UTR and 3D domain (21, 26); however, RT-PCR and rRT-PCR assays targeting the 5'-UTR empirically have lower sensitivity. To enhance the sensitivity of RT-PCR, the primer set FM8/9 were designed to target a conserved region of the 3D domain (17). This primer set was used for nucleic acid analyses during 2000 and 2010 outbreaks in Japan; however, data of its performance including sensitivity against different topotypes of FMDVs and specificity compared with those of the 1F/R primer set has not been published. Therefore,

in the present study, the performance of RT-PCR using FM8/9 was compared with those of other RT-PCR assays.

# Materials and Methods

### *Cells*

Primary bovine kidney (BK), *Instituto Biologico rim suino* and pig kidney number 2 (IB-RS-2), baby hamster kidney (BHK-21), cloned porcine kidney (CPK) and Madin-Darby bovine kidney (MDBK) cells were grown in Eagle's Minimum Essential Medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan), while fetal goat tongue (ZZ-R 127) cells (27) were grown in Dulbecco's Modified Eagle's Medium (DMEM): Nutrient Mixture F-12 (Gibco-BRL, Palo Alto, CA, USA) supplemented with fetal bovine serum. The cells were maintained at 37°C in a 5% $CO_2$ atmosphere.

### *Viruses*

Virus isolation was performed according to the OIE Terrestrial Manual (19). The virus strains O/JPN/2010 and O/JPN/2000 were isolated from clinical samples obtained from cattle in Japan using BK cells and passaged three times in BHK-21 cells. O/MOG/4/2017 and O/MOG/2/2015, supplied by State Central Veterinary Laboratory (Mongolia), were propagated using ZZ-R 127 cells, and O/TAI/10/2016 and A/TAI/46-1/2015, supplied by Regional Reference Laboratory for FMD in South East Asia (Thailand) were propagated using BHK-21 cells. Another 18 FMDV strains, supplied by the Pirbright Institute (United Kingdom), swine vesicular disease virus (SVDV) J1'73, and two strains of vesicular stomatitis virus (VSV) of the New Jersey and Indiana 1 serotypes were grown on monolayers of IB-RS-2 and/or BHK-21 cells. The classical swine fever virus (CSFV)/JPN/2018 was isolated from clinical samples obtained from pigs in Japan and propagated using CPK cells, similar to the CSFV/ALD strain. The bovine rhinitis virus A (BRAV) strains SD-1, M-17, and H-1 and bovine rhinitis virus B (BRBV) strain EC11 were propagated using BK and

MDBK cells at 33°C in a 5% $CO_2$ atmosphere. Senecavirus A (SVA) isolated in the U.S.A. in 1993, 2007, 2011 and 2015 (strain No. 94-9356, 503297, 11-055910, 15-029085 and 15-031348), and African Swine Fever virus (ASFV) Kenia/05 and Armenia/07 were obtained from Plum Island Animal Disease Center (U.S.A.) and Complutense University of Madrid (Spain), respectively. All stock viruses were stored at –80°C.

***Virus titration and dilution***

Virus titration was performed as follows: serial tenfold dilutions of the stock viruses were prepared in tubes, and each dilution was inoculated into ten wells of the 96-well plates with the same volume of suspension of IB-RS-2 cells. The cultures were incubated for 72 hr at 37°C in 5% $CO_2$ and the appearance of cytopathic effect (CPE) was observed. Virus titers of tissue culture infectious dose ($TCID_{50}$) were calculated by the Reed-Muench method. For comparative analysis of the detection limits for each nucleic acid analysis, stock viruses were diluted ten-fold until $10^2$ $TCID_{50}$/0.1ml, and from $10^2$ $TCID_{50}$/0.1ml, stock viruses were further diluted four-fold.

***RNA extraction, reverse transcription-PCR and real-time RT-PCR***

The primer set FM8/9 was designed to amplify 644 nucleotides in a region in the 3D domain (Fig. 2). The 271, 108, 50, 28, 14, 15, and 8 sequences of the FMDV of serotype O, A, Asia 1, C, and SAT 1–3, respectively, which encompass the complete sequences of the 5'-UTR and 3D domain, were downloaded from GenBank and aligned using ClastalW in MEGA 7.0 software (28). Nucleotide variability at each position was assessed, and the target region of FM8/9 was defined as a highly conserved region in the FMDV genome (data not shown).

Viral RNA was extracted from supernatants of infected cells using the High Pure Viral

RNA Kit (Roche Diagnostics, Tokyo, Japan). FMDV-specific genes were detected using a Superscript III One-Step RT-PCR System with Platinum Taq Polymerase (Life Technologies) and 200 nM of primer set FM8/9 or 1F/R (Table 1). PCR amplification was performed using an ABI GeneAmp PCR System 9700 (Life Technologies) as follows: 55°C for 30 min, one cycle; 94°C for 2 min, one cycle; 94°C for 15 s, 55°C for 30 s, and 68°C for 45 s, 35 cycles; and 68°C for 5 min, one cycle. PCR products were separated using electrophoresis on 1% agarose gels. Amplicons were stained with ethidium bromide and visualized using UV-light transillumination (Fig. 3). Serial dilutions of each strain were tested in the same reaction and the reproducibility of weak positive results was confirmed by triplicate trials. The rRT-PCR assay was conducted using the TaqMan RNA-to-Ct 1-Step Kit (Life Technologies) with the 900 nM of primer sets 5'-UTR or 3D-Forward, Reverse, and 250 nM of Probe (Table 1), which are described in the OIE Terrestrial Manual (19). In the present study, samples with a Ct value below 37 were defined as positive.

***Experimental infections***

Two 2-month-old pigs were intradermally inoculated with 1 ml of $10^{5.3}$ $TCID_{50}$ of O/JPN/2010 and cohabited with 2 pigs each (29). Two 6-month-old Holstein cows were inoculated intradermally with 1 ml of $10^{7.5}$ $TCID_{50}$ of O/MOG/4/2017 and cohabited with one cow each. Sera and saliva samples were collected daily for 11 days post-infection (dpi) and 10 dpi, respectively. Blood for serum preparation was collected from a cervical vein using a vacuum blood collection tube (Venoject II, Terumo Corporation, Tokyo, Japan). Saliva was collected from the oral cavity using a roll-shaped synthetic saliva collector, Salivette (SARSTEDT AG & Co. KG, Nümbrecht, Germany) and forceps. Viral RNA was extracted from the samples as described above and analyzed using RT-PCR and rRT-PCR assays with the respective primer sets described above. The experimental infections were

authorized by the Animal Care and Use Committee of the and were performed in a high-containment facility at the National Institute of Animal Health (NIAH) (authorization number: 10-077 and 17-016).

# Results

### *Detection limits of RT-PCR using primer set FM8/9 or 1F/R*

To compare the detection limits of RT-PCR assays in which the FM8/9 and 1F/R primer sets were used to amplify the genes, RNAs of 10 and 9 FMDV strains each for serotypes O and A, and one strain each for serotypes Asia1, C, and SAT1-3 were extracted from each serial dilution of the FMDV strains and subjected to RT-PCR and rRT-PCR (Table 2). The nucleotide sequences of each primer's target region of the FMDV strains are shown in Table 3. The sensitivities of RT-PCR reactions primed using FM8/9 were >4-fold higher in the detection of 21 strains (gray shading in Table 2) than those using 1F/R. Moreover, compared to the rRT-PCR assay targeting the 3D domain, the sensitivities of RT-PCR assays using FM8/9 were >4-fold higher in the detection of 10 strains (dark gray shading in Table 2), but were lower in the detection of 5 strains (O/TUR/1/1978, A/TAI/2011, A/TAI/46-1/2015, A22 IRQ 24/1964 and SAT2/SAU/2000). In contrast, the sensitivities of rRT-PCR assays targeting the 5'-UTR were much lower than RT-PCR assays using FM8/9 for all strains tested (data not shown), suggesting that the performance of 5'-UTR-targeted rRT-PCR assays in the laboratories could be improved by optimizing the assay protocol.

### *Validation of the specificity of primer sets for non-FMDV viruses*

To validate specificity, RNA samples were extracted from supernatants of cells infected with SVDV strain J1'73; VSV strains of New Jersey and Indiana 1 serotypes; BRAV strains SD-1, M-17, and H-1; BRBV strain EC11; SVA strains isolated in the U.S.A. in 1993, 2007, 2011 and 2015; CSFV strains CSFV/JPN/2018 and ALD; and ASFV strains Kenia/05 and Armenia/07. RNA was subjected to RT-PCR using each primer set and to rRT-PCR. There were no false-positive reactions in the RT-PCR assay using 1F/R or rRT-PCR targeting the

5’-UTR and 3D domain. RNAs from SVDV, VSV, BRBV, SVA, CSFV and ASFV were not detectably amplified by the RT-PCR assay using FM8/9, but RNA from the BRAV strain SD-1 was weakly detected (data not shown). These results were confirmed by triplicate trials. Both nucleotide sequence at the 3’ end of the FM8/9 target region of this strain match with the primers, although three mismatches were confirmed near the 3’ terminus of FM9 (Table 3).

***Detection of FMDV genes from clinical samples using RT-PCR***

To assess the validity of the RT-PCR methods for testing clinical samples, sera and saliva samples from infected and contact pigs and cows from previous experimental infection studies were analyzed using the RT-PCR and rRT-PCR assays with the respective primer sets (Tables 4 and 5). The RT-PCR assay using FM8/9 detected positives from 1 dpi to 10 dpi and 1 day post-contact (dpc) to 10 dpc in inoculated and contact pigs, respectively. In contrast, FMDV genes were detected from 1 dpi to 8 dpi and 2 dpc to 9 dpc using 1F/R. For samples from inoculated and contact cows, the RT-PCR assay using FM8/9 detected FMDV genes from 1 dpi to 9 or 10 dpi, and from 2 dpc to 9 or 10 dpc, respectively. The RT-PCR assay using 1F/R detected FMDV genes from 1 dpi to 5 or 10 dpi, or from 4 dpi to 10 dpi, and from 2 dpc to 7 dpc, respectively. These findings indicate that the RT-PCR assay using FM8/9 detected FMDV genes for a longer period from all infected pigs and cows than that using 1F/R. In addition, the rRT-PCR assay targeting the 5’-UTR detected genes for a limited period of infection. Therefore, RT-PCR using FM8/9 achieved higher relative sensitivity for clinical samples from infected pigs and cows. In contrast, the rRT-PCR assay targeting the 3D domain detected FMDV genes from all samples that were positive in the FM8/9 assay, and was additionally positive for seven and three samples from pigs and cows, respectively, which were negative in the FM8/9 assay.

## Discussion

In the present study, it was demonstrated that the RT-PCR assay using the primer set FM8/9 targeting the conserved 3D region of FMDV has much higher sensitivity for FMDVs and clinical samples from cattle and pigs than RT-PCR assays targeting the 5'-UTR. Moreover, compared to the rRT-PCR assay, the sensitivities of established RT-PCR assays were higher in the detection of 10 out of 24 strains. However, it is regrettable that only a limited number of strains, especially for serotype Asia1, C, and SAT1-3, could be used in this study to validate the PCR assays. In addition, the rRT-PCR assay targeting the 3D domain showed even higher sensitivity for clinical samples. However, because the rRT-PCR assay is associated with the risk of nonspecific signals as mentioned above and showed apparently lower sensitivity than the RT-PCR assay for several strains in this study, it should be used along with other methods for diagnosing FMD. Although extensive validation using a much wider range of isolates, especially from Africa, should be performed in the future, the RT-PCR assay using FM8/9 clearly demonstrated high sensitivity for FMDVs in this study.

Although it was assumed that the low sensitivity of the RT-PCR assay using 1F/R to detect SAT3/ZIM/1983 could be explained by two or more consecutive mismatches in the nucleotide sequences of the target regions of the 1F/R primers (Table 3), such a significant difference in identities between the sequences of the two primer sets among the analyzed FMDV strains were not found. These findings indicate that the sensitivities of the RT-PCR assays using each primer set may not only depend on identical nucleotide sequences in the target region of each primer. Rather, the Tm values and GC-contents of the primer sets, efficiency of the amplification of the sequence between the primer target regions could influence sensitivities of the RT-PCR (30). The higher-order structures of 5'-UTR of

FMDV may also be a disadvantage for the efficiency of reverse transcription reaction in this region (2).

In this study, RNA from the BRAV strain SD-1 was weakly amplified by the RT-PCR assay using FM8/9, suggesting that diagnosis using only the RT-PCR assay with FM8/9 could lead to misidentification. However, the present RT-PCR method did not show false-positive reaction for sera, saliva, or nasal samples obtained from healthy 32 Holstein cows (age >3 months) in experimental infections conducted in our institute (data not shown). Furthermore, the diagnosis of FMD in practice is determined using RT-PCR, rRT-PCR, virus isolation, nucleotide sequencing, serological tests, and epidemiological data, including clinical signs. One clinical sign of BRV is mild respiratory disease (30, 31). Moreover, rRT-PCR, which was highly sensitive for detecting FMDV genes, did not detect BRAV, suggesting that it would not be difficult to differentiate between these viruses based on several methods to diagnose FMD.

Accurate diagnosis of economically important diseases such as FMD requires the use of multiple PCR methods along with other diagnostics. The present study describes a method that will likely contribute to the efforts to more accurately detect FMDV. While limited numbers of strains, especially of serotypes Asia1, C, and SAT1-3, were analyzed in this study, the assay was demonstrated to have much higher sensitivity for FMDVs and clinical samples from cattle and swine than RT-PCR assays targeting the 5'-UTR. These results suggest that PCR assays that incorporate primer set FM8/9 may have higher sensitivity for detecting FMDV genes and are therefore suitable for the diagnosis of FMD.

## Summary

The potential of FMD to spread extensively means that rapid and accurate methods are needed for its diagnosis. Therefore, RT-PCR plays an important diagnostic role. The primer set FM8/9 were designed to amplify 644 bases in the conserved 3D region of all seven serotypes of the FMDV. Here, the performance of RT-PCR assays using FM8/9 was compared with those using the primer set 1F/R, which targets the 5'-UTR, and rRT-PCR assays described in the OIE manual. Detection limits of the RT-PCR assays were determined for 24 strains, representing all serotypes. The sensitivities of RT-PCR assays using FM8/9 were $10^{0.6}$- to $10^{3.8}$-fold higher than those of 1F/R assays for 21 strains. To assess the validity of the methods for analyzing clinical samples, sera and saliva samples collected daily from pigs and cows infected with FMDV were analyzed using the four PCR assays. FM8/9 assays detected FMDV from all infected pigs and cows for longer periods than 1F/R assays, indicating that FM8/9 assays have higher sensitivity for the clinical samples. These results suggest that the FM8/9 RT-PCR assay is highly sensitive and is therefore suitable for the diagnosis of FMD.

**Table 1.** Primers for detecting FMDV genes using RT-PCR

| Primer | Sequence (5'-3')[a] | Target region | Size of product (bp) | Reference |
|---|---|---|---|---|
| FM8 | TGTCAGACCTTCCTGAAGGACG | 3D | 644 | |
| FM9 | CCTTTGTCGCTTTTGTCAGCTGG | | | |
| 1F | GCCTGGTCTTTCCAGGTCT | 5'-UTR | 328 | (25) |
| 1R | CCAGTCCCCTTCTCAGATC | | | |
| 3D Forward | ACTGGGTTTTACAAACCTGTGA | 3D | 107 | (26) |
| 3D Reverse | GCGAGTCCTGCCACGGA | | | |
| 3D Probe | TCCTTTGCACGCCGTGGGAC | | | |
| 5'-UTR Forward | CACYTYAAGRTGACAYTGRTACTGGTAC | 5'-UTR | 97 | (21) |
| 5'-UTR Reverse | CAGATYCCRAGTGWCICITGTTA | | | |
| 5'-UTR Probe | CCTCGGGGTACCTGAAGGGCATCC | | | |

[a] The following sequence ambiguity code was used: R (A/G), W (A/T), Y (C/T).

**Table 2.** Detection limits of RT-PCR using each primer set

| | Detection limit ($TCID_{50}$/0.1ml)[a] | | |
|---|---|---|---|
| | RT-PCR | | rRT-PCR[b] |
| Virus strain | FM8/9 | 1F/R | 3D F/R |
| O/JPN/2010 | $10^{-1.0}$ | $10^{-0.4}$ | $10^{-1.0}$ |
| O/TUR/5/2009 | $10^{-1.0}$ | $10^{0.2}$ | $10^{0.2}$ |
| O/MOG/4/2017 | $10^{-1.6}$ | $10^{-1.0}$ | $10^{-1.6}$ |
| O/ECU/4/2010 | $10^{-1.0}$ | $10^{0.2}$ | $10^{0.8}$ |
| O/HKN/2015 | $10^{0.2}$ | $10^{0.8}$ | $10^{0.2}$ |
| O/JPN/2000 | $10^{-0.4}$ | $10^{0.8}$ | $10^{0.8}$ |
| O/TAW/1/2013 | $10^{-1.6}$ | $10^{1.4}$ | $10^{-1.0}$ |
| O/TAI/10/2016 | $10^{-2.2}$ | $10^{0.2}$ | $10^{-1.0}$ |
| O/TUR/1/1978 (Manisa) | $10^{0.2}$ | $10^{0.2}$ | $10^{-0.4}$ |
| O/MOG/2/2015 | $10^{-1.0}$ | $10^{-0.4}$ | $10^{-1.0}$ |
| A/TAI/2011 | $10^{0.2}$ | $10^{-0.4}$ | $10^{-1.0}$ |
| A/TAI/46-1/2015 | $10^{-0.4}$ | $10^{0.2}$ | $10^{-1.0}$ |
| A/SAU/15/2016 | $10^{-1.0}$ | $10^{0.8}$ | $10^{0.8}$ |
| A/IRN/1/2011 | $10^{-0.4}$ | $10^{0.2}$ | $10^{-0.4}$ |
| A22 IRQ 24/1964 | $10^{0.2}$ | $10^{1.4}$ | $10^{-0.4}$ |
| A/MOG/13/2013 | $10^{-0.4}$ | $10^{0.8}$ | $10^{0.2}$ |
| A/MAY/1997 | $10^{-2.2}$ | $10^{-0.4}$ | $10^{-1.6}$ |
| A/IRN/1/2016 | $10^{-1.0}$ | $10^{0.2}$ | $10^{-1.0}$ |
| A/Argentina/2001 | $10^{-2.8}$ | $10^{-0.4}$ | $10^{-1.6}$ |
| Asia1/TUR/2011 | $10^{-1.0}$ | $10^{0.2}$ | $10^{-1.0}$ |
| C/PHI/7/1984 | $10^{-0.4}$ | $10^{0.2}$ | $10^{0.8}$ |
| SAT1/KEN/2009 | $10^{0.2}$ | $10^{3}$ | $10^{0.2}$ |
| SAT2/SAU/2000 | $10^{0.8}$ | $10^{-0.4}$ | $10^{-1.0}$ |
| SAT3/ZIM/1983 | $10^{0.2}$ | $10^{4}$ | $10^{0.2}$ |

[a] Boxes indicating the primer set that achieved the highest sensitivity for detecting each virus strain are shaded in light gray. Boxes showing higher sensitivity using RT-PCR with FM8/9 compared with rRT-PCR are shaded in dark gray.
[b] Samples with a Ct value below 37 were defined as positive.

**Table 3.** Comparison of nucleotide sequences of each primer target region of FMDVs and BRAV

| | FM8 | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Virus strain | t | g | t | c | a | g | a | c | c | t | t | c | c | t | g | a | a | g | g | a | c | g |
| O/JPN/2010 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TUR/5/2009 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/MOG/4/2017 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/ECU/4/2010 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/HKN/2015 | . | . | C | . | . | A | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . |
| O/JPN/2000 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TAW/1/2013 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TAI/10/2016 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TUR/1/1978 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | . | . | . | . |
| O/MOG/2/2015 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/TAI/2011 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | . | . | . | . |
| A/TAI/46-1/2015 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/SAU/15/2016 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/IRN/1/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A22 IRQ 24/1964 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/MOG/13/2013 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/MAY/1997 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/IRN/1/2016 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/Argentina/2001 | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Asia1/TUR/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| C/PHI/7/1984 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| SAT1/KEN/2009 | . | . | C | . | . | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| SAT2/SAU/2000 | . | . | C | . | . | A | . | . | A | . | . | . | . | . | . | . | . | . | . | . | . | . |
| SAT3/ZIM/1983 | . | . | . | . | . | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| BRAV SD-1 | . | . | . | . | . | . | . | . | T | . | . | T | . | . | . | . | . | . | . | . | . | . |

"." indicates the same nucleotide in each primer. Nonidentical bases are written in uppercase.

**Table 3.** (Continued)

| | **FM9**[a] | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Virus strain | c | c | a | g | c | t | g | a | c | a | a | a | a | g | c | g | a | c | a | a | a | g | g |
| O/JPN/2010 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TUR/5/2009 | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/MOG/4/2017 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| O/ECU/4/2010 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/HKN/2015 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/JPN/2000 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TAW/1/2013 | . | . | G | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TAI/10/2016 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| O/TUR/1/1978 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/MOG/2/2015 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| A/TAI/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/TAI/46-1/2015 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| A/SAU/15/2016 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| A/IRN/1/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A22 IRQ 24/1964 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/MOG/13/2013 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| A/MAY/1997 | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . |
| A/IRN/1/2016 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| A/Argentina/2001 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Asia1/TUR/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| C/PHI/7/1984 | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| SAT1/KEN/2009 | . | . | . | . | . | C | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| SAT2/SAU/2000 | . | . | . | . | . | C | . | . | . | . | . | . | . | . | T | . | . | . | . | G | . | . | . |
| SAT3/ZIM/1983 | . | . | . | . | . | C | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . |
| BRAV SD-1 | . | . | T | . | A | A | . | . | . | . | . | . | . | . | . | . | . | . | . | G | G | . | . |

"." indicates the same nucleotide in each primer. Nonidentical bases are written in uppercase.

[a] Reverse complement of FM9 and 1R sequences are indicated.

**Table 3.** (Continued)

| | 1F | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Virus strain | g | c | c | t | g | g | t | c | t | t | t | c | c | a | g | g | t | c | t |
| O/JPN/2010 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TUR/5/2009 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/MOG/4/2017 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/ECU/4/2010 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/HKN/2015 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/JPN/2000 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TAW/1/2013 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TAI/10/2016 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TUR/1/1978 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/MOG/2/2015 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/TAI/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/TAI/46-1/2015 | . | . | . | . | . | . | . | . | A | . | . | . | . | . | . | . | . | . | . |
| A/SAU/15/2016 | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . |
| A/IRN/1/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A22 IRQ 24/1964 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/MOG/13/2013 | . | . | . | . | . | . | . | . | A | . | . | . | . | . | . | . | . | . | . |
| A/MAY/1997 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/IRN/1/2016 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/Argentina/2001 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Asia1/TUR/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| C/PHI/7/1984 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| SAT1/KEN/2009 | . | . | . | C | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . |
| SAT2/SAU/2000 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| SAT3/ZIM/1983 | . | . | . | C | A | . | . | . | . | . | . | . | T | G | . | . | . | . | . |

"." indicates the same nucleotide in each primer. Nonidentical bases are written in uppercase.

**Table 3.** (Continued)

| | **1R**[a] | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Virus strain | g | a | t | c | t | g | a | g | a | a | g | g | g | g | a | c | t | g | g |
| O/JPN/2010 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . |
| O/TUR/5/2009 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/MOG/4/2017 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/ECU/4/2010 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/HKN/2015 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . |
| O/JPN/2000 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/TAW/1/2013 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . |
| O/TAI/10/2016 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . |
| O/TUR/1/1978 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| O/MOG/2/2015 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/TAI/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . |
| A/TAI/46-1/2015 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/SAU/15/2016 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/IRN/1/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A22 IRQ 24/1964 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | A |
| A/MOG/13/2013 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/MAY/1997 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/IRN/1/2016 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| A/Argentina/2001 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . |
| Asia1/TUR/2011 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| C/PHI/7/1984 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | A | . |
| SAT1/KEN/2009 | . | . | . | . | . | . | . | . | . | . | . | . | A | . | . | . | C | . | . |
| SAT2/SAU/2000 | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . |
| SAT3/ZIM/1983 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | A | A |

"." indicates the same nucleotide in each primer. Nonidentical bases are written in uppercase.

[a] Reverse complement of FM9 and 1R sequences are indicated.

**Table 4.** Detection of clinical samples of FMD obtained from pigs inoculated with FMDV O/JPN/2010 and their uninoculated cohabitants

| Pig No. | Sample | Primer set | Days post-inoculation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| | | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 (inoculated) | Serum | FM8/9[a] | – | + | + | + | – | – | – | – | – | – | – | – |
| | | 1F/R | – | + | + | – | – | – | – | – | – | – | – | – |
| | | 3D F/R | – | + | + | + | + | – | – | – | – | – | – | – |
| | | 5'-UTR F/R | – | + | + | – | – | – | – | – | – | – | – | – |
| | Saliva | FM8/9 | – | + | + | + | + | + | + | + | + | + | + | – |
| | | 1F/R | – | + | + | + | + | + | + | + | + | – | – | – |
| | | 3D F/R | – | + | + | + | + | + | + | + | + | + | + | – |
| | | 5'-UTR F/R | – | + | + | + | + | + | + | + | – | – | – | – |
| 2 (inoculated) | Serum | FM8/9 | – | + | + | + | – | – | – | – | – | – | – | – |
| | | 1F/R | – | + | + | + | – | – | – | – | – | – | – | – |
| | | 3D F/R | – | + | + | + | + | + | – | – | – | – | – | – |
| | | 5'-UTR F/R | – | + | + | + | – | – | – | – | – | – | – | – |
| | Saliva | FM8/9 | – | + | + | + | + | + | + | + | + | + | + | – |
| | | 1F/R | – | + | + | + | + | + | + | + | + | – | + | – |
| | | 3D F/R | – | + | + | + | + | + | + | + | + | + | + | + |
| | | 5'-UTR F/R | – | + | + | + | + | + | + | – | – | – | – | – |
| 3 (contact) | Serum | FM8/9 | NS[b] | – | – | + | + | + | – | – | – | – | – | – |
| | | 1F/R | | – | – | + | + | + | – | – | – | – | – | – |
| | | 3D F/R | | – | – | + | + | + | – | – | – | – | – | – |
| | | 5'-UTR F/R | | – | – | + | + | + | – | – | – | – | – | – |
| | Saliva | FM8/9 | NS | – | + | + | + | + | + | + | + | + | + | + |
| | | 1F/R | | – | – | + | + | + | + | + | + | + | + | – |
| | | 3D F/R | | – | + | + | + | + | + | + | + | + | + | + |
| | | 5'-UTR F/R | | – | – | – | + | + | – | + | – | – | – | – |

[a] Results of RT–PCR assays using FM8/9 in this Table are published (29).

[b] NS, not sampled

**Table 4.** (Continued)

| Pig No. | Sample | Primer set | Days post-inoculation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| | | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 4 (contact) | Serum | FM8/9 | NS | – | + | + | + | + | – | – | – | – | – | – |
| | | 1F/R | | – | – | + | + | + | – | – | – | – | – | – |
| | | 3D F/R | | – | + | + | + | + | – | – | – | – | – | – |
| | | 5’-UTR F/R | | – | – | + | + | + | – | – | – | – | – | – |
| | Saliva | FM8/9 | NS | – | + | + | + | + | + | + | + | + | – | – |
| | | 1F/R | | – | – | + | + | + | – | + | + | + | – | – |
| | | 3D F/R | | – | + | + | + | + | + | + | + | + | + | + |
| | | 5’-UTR F/R | | – | – | + | + | + | – | – | – | – | – | – |
| 5 (contact) | Serum | FM8/9 | NS | – | – | – | – | + | + | + | + | – | – | – |
| | | 1F/R | | – | – | – | – | + | + | + | – | – | – | – |
| | | 3D F/R | | – | – | – | – | + | + | + | + | + | – | – |
| | | 5’-UTR F/R | | – | – | – | – | + | + | + | – | – | – | – |
| | Saliva | FM8/9 | NS | – | + | + | + | + | + | + | + | + | + | – |
| | | 1F/R | | – | – | – | – | + | + | + | + | + | – | – |
| | | 3D F/R | | – | + | + | + | + | + | + | + | + | + | + |
| | | 5’-UTR F/R | | – | – | – | – | + | + | + | + | + | – | – |
| 6 (contact) | Serum | FM8/9 | NS | – | + | + | + | + | – | – | – | – | – | – |
| | | 1F/R | | – | + | + | + | + | – | – | – | – | – | – |
| | | 3D F/R | | – | + | + | + | + | + | + | + | – | – | – |
| | | 5’-UTR F/R | | – | + | + | + | – | – | – | – | – | – | – |
| | Saliva | FM8/9 | NS | – | + | + | + | + | + | + | + | + | + | + |
| | | 1F/R | | – | – | + | + | + | + | + | + | + | – | – |
| | | 3D F/R | | – | + | + | + | + | + | + | + | + | + | + |
| | | 5’-UTR F/R | | – | – | + | + | + | + | – | – | – | – | – |

[a] Results of RT–PCR assays using FM8/9 in this Table are published (29).

[b] NS, not sampled

**Table 5.** Detection of FMDV in clinical samples obtained from cattle inoculated with FMDV O/MOG/4/2017 and contact animals

| Cow No. | Sample | Primer set | Days post-inoculation | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 (inoculated) | Serum | FM8/9 | – | + | – | + | + | – | – | – | – | – | – |
| | | 1F/R | – | + | – | + | – | – | – | – | – | – | – |
| | | 3D F/R | – | + | – | + | + | – | – | + | – | – | – |
| | | 5’-UTR F/R | – | + | – | + | + | – | – | – | – | – | – |
| | Saliva | FM8/9 | – | + | + | + | + | + | – | + | – | + | – |
| | | 1F/R | – | + | + | + | + | + | – | – | – | – | – |
| | | 3D F/R | – | + | + | + | + | + | – | + | + | + | – |
| | | 5’-UTR F/R | – | + | + | + | + | – | – | – | – | – | – |
| 2 (in contact with Cow No. 1) | Serum | FM8/9 | – | – | – | + | + | + | + | + | – | – | – |
| | | 1F/R | – | – | – | – | + | + | + | – | – | – | – |
| | | 3D F/R | – | – | – | + | + | + | + | + | – | – | – |
| | | 5’-UTR F/R | – | – | – | – | + | + | – | – | – | – | – |
| | Saliva | FM8/9 | – | – | + | + | + | + | + | + | + | + | + |
| | | 1F/R | – | – | – | – | + | + | + | + | + | + | + |
| | | 3D F/R | – | – | – | + | + | + | + | + | + | + | + |
| | | 5’-UTR F/R | – | – | – | + | + | + | – | – | – | – | – |
| 3 (inoculated) | Serum | FM8/9 | – | + | + | + | + | + | – | + | – | – | – |
| | | 1F/R | – | + | + | + | + | – | – | – | – | – | – |
| | | 3D F/R | – | + | + | + | + | + | – | + | – | – | – |
| | | 5’-UTR F/R | – | – | + | + | – | – | – | – | – | – | – |
| | Saliva | FM8/9 | – | + | + | + | + | + | + | + | + | + | + |
| | | 1F/R | – | + | + | + | + | + | + | + | + | + | + |
| | | 3D F/R | – | + | + | + | + | + | + | + | + | + | + |
| | | 5’-UTR F/R | – | + | + | + | – | – | – | – | – | – | – |
| 4 (in contact with Cow No. 3) | Serum | FM8/9 | – | – | + | + | + | + | + | + | – | – | – |
| | | 1F/R | – | – | + | – | + | + | + | – | – | – | – |
| | | 3D F/R | – | – | + | – | + | + | + | + | – | – | – |
| | | 5’-UTR F/R | – | – | + | – | – | + | – | – | – | – | – |
| | Saliva | FM8/9 | – | – | – | + | + | + | + | + | + | + | – |
| | | 1F/R | – | – | – | – | + | + | + | + | – | – | – |
| | | 3D F/R | – | – | – | – | + | + | + | + | + | + | + |
| | | 5’-UTR F/R | – | – | – | – | + | – | – | – | – | – | – |

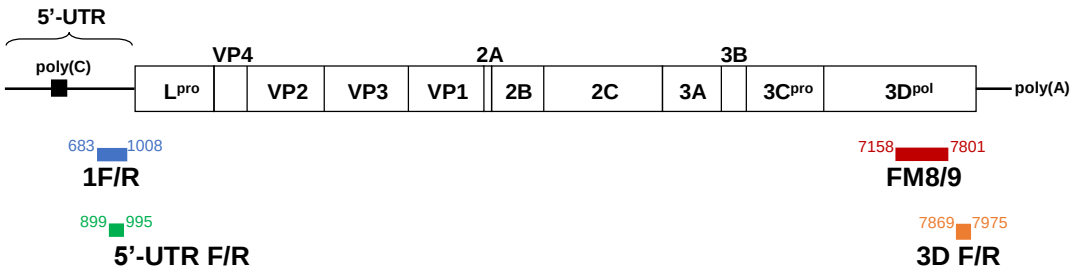


**Fig. 2. Location of target regions using RT-PCR and rRT-PCR within the 5'-UTR and 3D domain of FMDV.**

The target regions of 1F/R, FM8/9, and primer and probe sets for rRT-PCR are indicated. Nucleotide position of the amplified fragment by each PCR assay were indicated corresponds to the nucleotide sequence of O/JPN/2010 290-1E (Genbank accession No. LC036265) .

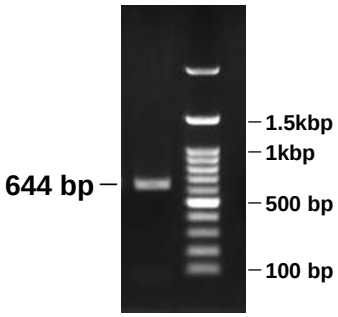


**Fig. 3. Amplicon of FMDV amplified using RT-PCR with the FM8/9 primer set.** RNA was extracted from the supernatant of BHK-21 cells infected with O/JPN/2010 and subjected to RT-PCR using the FM8/9 primer set. Amplicon (left) and 100bp DNA Ladder (Nippon Genetics, Tokyo, Japan) (right) were stained with ethidium bromide and visualized using UV-light transillumination.

# Chapter II

## Genome variability of foot-and-mouth disease virus during the short period of the 2010 epidemic in Japan

# Introduction

In April 2010, an FMD outbreak occurred in Miyazaki Prefecture, in the south of Japan (18). After the confirmation of the first case on April 20th, the epidemic continued until July 4th and involved a total of 292 farms and over 200,000 cattle, swine, sheep and goats, and about 290,000 animals have been culled, including vaccinated animals. During the epidemic, the Japanese eradication strategy was implemented, which includes stamping out, movement restrictions and disinfection of contaminants. Finally, the epidemic was suppressed after a total of 76 days in one prefecture. A virus isolate from the first case, O/JPN/1/2010 (GenBank accession no. KF112885), was phylogenetically analyzed using sequences of the VP1 region, and classified into genotype Mya-98 of topotype Southeast Asia (8). In addition, from the results of experimental infections, this virus isolate was confirmed to be pathogenic in swine, cattle and goats, and to spread efficiently by direct contact (29, 32).

As with other RNA viruses, the FMDV genome has the high mutation rate of $10^{-3}$ to $10^{-5}$ per genomic replication, leading to enormous genetic variation due to poor fidelity of RNA replication (23). Several genetic analyses of FMDV have appeared, including sequence variability of FMDVs isolated from several areas and years (33-35), comparative analysis of FMDVs representing seven different serotypes (36), and molecular epidemiological analysis of sporadic outbreaks (37, 38). To date, however, few studies have described the genome dynamics of the whole L-fragment gene of FMDV over a short period and limited scale of an epidemic after the first introduction of FMDV into an FMD-free area, such as Japan.

Here, how FMDVs genetically evolved during the short period of the 2010 epidemic in Japan were analyzed. Virus isolates obtained from each case were subjected to

phylogenetic analysis, and their whole L-fragment genes which is composed of whole ORF and part of 5'- and 3'UTR were comparatively analyzed.

# Materials and Methods

### *Cells and viruses*

BK and IB-RS-2 cells were grown in MEM (Nissui Pharmaceutical), while LFBK-αvβ6 cells (39, 40) and ZZ-R 127 cells (27) were grown in DMEM: nutrient mixture F-12 (Gibco-BRL) supplemented with fetal bovine serum. The cells were maintained at 37°C in a 5% $CO_2$ atmosphere. Virus isolation from clinical samples of cattle, swine and buffalo of the 2010 epidemic was conducted using the BK or IB-RS-2 cells according to the OIE Manual (19). The virus isolates were passaged at most one time on LFBK-αvβ6 or ZZ-R 127 to obtain a high titer viral sample. Passage histories of them were indicated in Table 6.

### *Clinical samples*

Clinical samples were submitted by the Miyazaki Prefectural Government for diagnosis of FMD occurring in 2010 in Japan. These samples were collected by veterinarians in accordance with the guidelines of the Act on Domestic Animal Infectious Diseases Control, in which the veterinarian collects samples such as epithelium or swabs from a lesion and soaks them in 2 ml of PBS.

### *RNA isolation, PCR, and sequencing*

Viral RNA was extracted from the supernatant of infected cells using a High Pure Viral RNA Kit (Roche Diagnostics). First-strand cDNA synthesis was performed using the SuperScript III Reverse Transcriptase (Life Technologies) and FMDV-specific 2B331R primer (5'-GGCACGTGAAAGAGACTGGAGAG-3') and 3'-2010R primer (5'-TGGATAAAGGAAACGGGAAAAGC-3'). Full length of the L-fragment gene of approximately 7.7 kbs was amplified by polymerase chain reaction with PrimeSTAR Max

DNA Polymerase (TaKaRa, Shiga, Japan) and two primer sets: set 1 consisted of 5'-2010F primer (5'-CGTTAAAGGGAGGTAACCACAAG-3') and 2B331R primer and set 2 consisted of 2B217F primer (5'-ATGGCCGCTGTAGCAGCACGGTC-3') and 3'-2010R primer. Their nucleotide sequences were analyzed using the Ion PGM system (Life Technologies). These sequences have been deposited to GenBank (accession no. LC149617-LC149720). The following sequence ambiguity code was used if multiple nucleotides were detected more than 20% of coverage: K (T/G), M (A/C), R (A/G), S (C/G), W (A/T), Y (C/T), B (C/T/G), D (A/T/G), H (A/C/T), V (A/C/G), and N (A/C/G/T).

***Validation of variability of FMDV O/JPN/2010 on serial passage in cell culture***

To verify the effect of passage through cell culture, whole L-fragment sequences of a virus derived directly from an epithelium of a pig inoculated with O/JPN/2010-1/14C and viruses passaged through cell cultures by all patterns used in the present study were determined and compared. Experimental infections of animals were authorized by the Animal Care and Use Committee of the NIAH (authorization number: 13-084) and were performed in a high-containment facility at the NIAH.

***Genetic analysis***

For phylogenetic analysis, nucleotide sequences were aligned using ClastalW and analyzed together with those from the public database by the maximum-likelihood (ML) method using MEGA 7.0 software (28) and Bayesian Markov Chain Monte Carlo (MCMC) method using the BEAST program (41) based on the Tamura-Nei model. The reliability of phylogenetic inference at each branch node was estimated by the bootstrap method with 1,000 replications. Calculation of pairwise identity rates and invariant rates among sequences were performed using MEGA 7.0 and GENETYX Ver. 12 (GENETYX). To

analyze the effect of host species tropism, amino acid sequences of 74 viruses isolated from cattle were compared with consensus sequence of 29 viruses isolated from swine, and vice versa, and identity rates of each amino acid positions were calculated.

# Results

***Phylogenetic analysis of FMDV isolates using whole L-fragment gene***

As a result of virus isolation from clinical samples of animals collected between March 31st and Jun 11th, one hundred and four virus isolates were obtained (Table 6). L-fragment nucleotides of approximately 7.7 kbs were determined and phylogenetically analyzed with 14 closely related strains from other countries registered at GenBank by the ML and MCMC method (Fig. 4). Both results showed that the virus isolates of the 2010 epidemic in Japan were classified into a single group.

***Genome variability of the L-fragment of FMDV isolates during the epidemic***

To analyze how FMDVs genetically varied through the epidemic, L-fragment sequences of 104 virus isolates were compared each other and pairwise identity rates among them were calculated. Among these 104 virus isolates, no sequences were completely identical with each other, and no genetic deletion or insertion was observed. Pairwise identity rates of these 104 virus isolates ranged from 99.56% to 99.98% (average 99.83%) (Table 7). The mean rate of substitution of them was estimated to be $2.88 \times 10^{-5}$ per site per day using the BEAST program. In the analysis of host specific substitution, there was no amino acid position showed less than 50% of identity to the consensus sequence of viruses isolated from another species, therefore, specific substitution related to host species was not confirmed.

***Genome variability of protein-coding regions of FMDV isolates during the epidemic***

Nucleotide and amino acid sequences of each of the 12 protein-coding regions of the 104 virus isolates were compared and pairwise identity rates (totally 5,356 pairs) and

invariant rates of them were calculated (Table 7). For these 12 proteins, average identities of nucleotide sequences of VP4 and 2C were the highest, 99.93%, and invariant rates of them in both nucleotide and amino acid sequences were more than 96.0%. In contrast, average identity rate of VP2 and invariant rate of VP1 in amino acid sequences were the lowest, 99.46% and 89.20%, respectively (Table 7). To analyze variable amino acid positions of FMDV among the epidemic, full amino acid sequences of 104 virus isolates were compared with consensus sequence of them (Fig. 5). As a result, six amino acid positions were confirmed as comparatively variable positions with more than 7.7% (8 of 104 virus isolates) proportion of samples with substitutions of amino acids, No.17 of L, No.78-79, 132 and 182 of VP2, No.63 of 3C and No.158 of 3D (Table 8).

***Genome variability of FMDV O/JPN/2010 on passage in cell culture***

Whole L-fragment sequences of a virus derived directly from epithelium of a pig inoculated with O/JPN/2010-1/14C and viruses passed through cell cultures by all pattern used in the present study (Table 6) were determined and compared. Through the passage, no significant substitution through passage were observed, except for three selections from ambiguity nucleotid R (A/G) at nucleotide position No. 313 of VP3, M (A/C) at nucleotide position No. 399 of VP1, and Y (C/T) at nucleotide position No. 26 of 3D to a single nucleotide of A, C, and C, respectively. This result suggests that genetic substitutions in this study did not occur by passage through cell culture, but rather through animals during the epidemic.

## Discussion

In the present study, 104 virus isolates of FMDV were obtained from a single epidemic in Japan in 2010. L-fragment nucleotides of 104 virus isolates were phylogenetically analyzed with foreign strains and classified into a single group which was closely related to O/BY/CHA/2010 (Fig. 4). It indicated that there was a single introduction of FMDV from foreign countries into Japan through the epidemic. In addition, in the ML phylogenetic tree, the virus strain of O/JPN/2010-6/1S was the most closely related to the virus isolated from the neighboring country, with a nucleotide sequence identity rate of 99.41%, and was thus speculated to be the initial isolate of these 104 virus isolates. This speculation is consistent with an epidemiological survey of this epidemic previously reported (42). On the other hand, in the MCMC-tree, the virus strain of O/JPN/2010-1/14C was speculated to be the initial isolate (Fig. 4b). Further genome data of FMDVs from epidemic countries should be analyzed and published for more precise analysis of transboundary movements of virus.

Whole L-fragment sequences of the 104 virus isolates were aligned and confirmed to include no genetic deletions or insertions. Although pairwise identity rates among them were more than 99.56% (Table 7), no sequences were completely identical with each other. In addition, ambiguity nucleotides were confirmed on the genome of most of virus isolates. These results indicated that genetic substitutions of the virus isolates had occurred not drastically during the epidemic but gradually and constantly over this period. This genetic variability was presumably induced by the high mutation rate of FMDV during replication and transmission in the course of the epidemic.

Identity rates and invariant rates of each of 12 protein-coding regions of the 104 virus isolates were calculated (Fig. 5, Table 7). Among these 12 proteins, VP4 and 2C were

confirmed to be the most highly conserved regions in the 2010 epidemic (Table 7). VP4 is one of the structural proteins holding viral structural conformation within the capsid and is cleaved from VP0 together with VP2 in the final step of capsid assembly (2). The 2C region plays an important role in membrane binding during viral replication (43), and has ATPase and GTPase activities (44). These protein-coding regions have been previously reported to be highly conserved (36), consistent with the present results, and speculated to need to be conserved for efficient transmission among the epidemic.

The poor fidelity of RNA-dependent RNA polymerase is an important factor of RNA virus biology. It provides a source of sequence diversity that allow virus quasispecies to form and enables the virus to adapt to changing environments (45), although it can also lead to the generation of nonviable templates. In this study, six amino acid positions were confirmed as comparatively variable positions with substitutions of amino acids, No.17 of L, No.78-79, 132 and 182 of VP2, No.63 of 3C and No.158 of 3D (Table 8). No.78-79 and 132 of VP2 are expected to be on the antigenic site 2 and reported as highly variable region (4). In addition, No.79 of VP2 is reported to be associated with persistent infection in cattle (46). High variety of amino acid substitution at No.17 of L and high fixed substitution at No.63 of 3C were confirmed (Table 8). These amino acids substitutions were confirmed in more than 8 of 104 virus isolates, suggesting they could be viable mutations for the virus, enable adaptation to environments in the field, and contribute efficient transmission through the epidemics.

VP1 is the most studied FMDV protein due to its significance for virus attachment and entry, protective immunity, and serotype specificity (2-6). The previous report about comparative analysis of FMDVs representing seven serotypes indicated that pairwise identity of VP1 was the lowest compared with other protein-coding regions (36). On the other hand, in the present study, any amino acid positions of VP1 was not confirmed as

highly variable. Similarly, other reports have indicated that substitutions in the VP1 region were no more frequent than in other parts of the genome through *in vivo* passages and epidemics (37, 47). These data suggest that genetic substitutions in the VP1 region were not fixed on defined position but occurred scatteredly through passages in animals.

In the present study, specific substitution related to host species was not confirmed. In addition, extensive mutations reported to be responsible for pathogenicity of FMDV such as a part of deletion in the 3A region which significantly contribute to pathogenicity in swine and cattle was not confirmed (9). Until the 2010 epidemic, Japan had remained FMD-free since 2000, and in the 2010 epidemic, all infected, suspected and vaccinated animals were culled as soon as FMD was confirmed. Therefore, the selection pressure of transmission across hosts and immunity induced by infection or vaccination were presumably limited in this epidemic. The rapid eradication strategy mentioned above was assumed to have contributed to suppress the scale of the epidemic and, as a result, prevented generation of an extensive mutant virus. By comparing these data with those of epidemics in endemic areas with high selective pressure, it would be elucidated how selective pressure influences the genetic variability of FMDV.

In the present study, genome variability of FMDV during the short period of the 2010 epidemic in Japan was analyzed by phylogenetic and comparative analyses using whole L-fragment genes of virus isolates obtained from each case. These data should provide valuable information about the genome dynamics of the highly contagious RNA virus like FMDV, and enable more precise analysis of transmission pathways combined with the data of epidemiological survey.

## Summary

FMDV is highly contagious and has a high mutation rate, leading to extensive genetic variation. To investigate how FMDV genetically evolves over a short period of an epidemic after initial introduction into an FMD-free area, whole L-fragment sequences of 104 FMDVs isolated from the 2010 epidemic in Japan, which continued for less than three months were determined and phylogenetically and comparatively analyzed. Phylogenetic analysis of whole L-fragment sequences showed that these isolates were classified into a single group, indicating that FMDV was introduced into Japan in the epidemic via a single introduction. Nucleotide sequences of 104 virus isolates showed more than 99.56% pairwise identity rates without any genetic deletion or insertion, although no sequences were completely identical to each other. These results indicate that genetic substitutions of FMDV occurred gradually and constantly during the epidemic and the generation of an extensive mutant virus could have been prevented by rapid eradication strategy. From comparative analysis of variability of each FMDV protein-coding region, VP4 and 2C regions showed the highest average identity rates and invariant rates, and were confirmed as highly conserved. In contrast, the protein-coding regions VP2 and VP1 were confirmed to be highly variable regions with the lowest average identity rates and invariant rates, respectively. These data demonstrate the importance of rapid eradication strategy in an FMD epidemic and provide valuable information on the genome variability of FMDV during the short period of an epidemic.

**Table 6.** Virus isolates obtained from the 2010 epidemic in Japan in the present study.

| Isolate | Date of sampling | Species | Passage history[a] | GenBank accession no. |
|---|---|---|---|---|
| O/JPN/2010-1/14C | April, 09 | cattle | BK1 | LC149617 |
| O/JPN/2010-2/5S | April, 20 | cattle | BK1 | LC149618 |
| O/JPN/2010-3/2S | April, 20 | cattle | BK1 | LC149619 |
| O/JPN/2010-5/6S | April, 22 | cattle | BK1 | LC149620 |
| O/JPN/2010-6/1S | March, 31 | buffalo | BK1LF1 | LC149621 |
| O/JPN/2010-8/1S | April, 24 | cattle | BK1 | LC149622 |
| O/JPN/2010-9/1S | April, 27 | cattle | BK1 | LC149623 |
| O/JPN/2010-10/396V | April, 27 | swine | BK1LF1 | LC149624 |
| O/JPN/2010-11/4S | April, 27 | cattle | BK1 | LC149625 |
| O/JPN/2010-15/1S | April, 28 | cattle | BK1 | LC149626 |
| O/JPN/2010-17/593F | April, 29 | swine | BK1 | LC149627 |
| O/JPN/2010-18/3S | April, 30 | swine | BK1 | LC149628 |
| O/JPN/2010-21/3S | April, 30 | cattle | BK1LF1 | LC149629 |
| O/JPN/2010-22/3S | May, 01 | swine | BK1 | LC149630 |
| O/JPN/2010-23/2S | May, 02 | cattle | BK1 | LC149631 |
| O/JPN/2010-27/3S | May, 03 | swine | BK1LF1 | LC149632 |
| O/JPN/2010-28/2S | May, 04 | swine | BK1 | LC149633 |
| O/JPN/2010-29/4S | May, 04 | swine | BK1 | LC149634 |
| O/JPN/2010-39/2S | May, 05 | swine | BK1LF1 | LC149635 |
| O/JPN/2010-46/3S | May, 06 | cattle | BK1LF1 | LC149636 |
| O/JPN/2010-47/3S | May, 06 | swine | BK1LF1 | LC149637 |
| O/JPN/2010-50/1S | May, 06 | cattle | BK1LF1 | LC149638 |
| O/JPN/2010-55/1S | May, 07 | swine | BK1LF1 | LC149639 |
| O/JPN/2010-61/1S | May, 07 | cattle | BK1LF1 | LC149640 |
| O/JPN/2010-65/3S | May, 08 | swine | BK1LF1 | LC149641 |
| O/JPN/2010-68/3S | May, 08 | cattle | BK1LF1 | LC149642 |
| O/JPN/2010-72/3S | May, 09 | swine | IB1LF1 | LC149643 |
| O/JPN/2010-82/1S | May, 09 | cattle | IB1LF1 | LC149644 |
| O/JPN/2010-83/2S | May, 09 | cattle | IB1LF1 | LC149645 |
| O/JPN/2010-84/1S | May, 09 | swine | IB1LF1 | LC149646 |
| O/JPN/2010-85/1S | May, 10 | cattle | IB1LF1 | LC149647 |
| O/JPN/2010-86/1S | May, 10 | cattle | BK1LF1 | LC149648 |
| O/JPN/2010-87/1S | May, 10 | swine | BK1LF1 | LC149649 |
| O/JPN/2010-88/5S | May, 10 | cattle | BK1LF1 | LC149650 |

**Table 6.** (Continued)

| Isolate | Date of sampling | Species | Passage history | GenBank accession no. |
|---|---|---|---|---|
| O/JPN/2010-90/5S | May, 11 | cattle | BK1LF1 | LC149651 |
| O/JPN/2010-92/3S | May, 11 | swine | BK1LF1 | LC149652 |
| O/JPN/2010-93/2S | May, 11 | swine | BK1LF1 | LC149653 |
| O/JPN/2010-94/5S | May, 11 | cattle | BK1LF1 | LC149654 |
| O/JPN/2010-98/1S | May, 12 | cattle | BK1LF1 | LC149655 |
| O/JPN/2010-100/5S | May, 12 | cattle | BK1LF1 | LC149656 |
| O/JPN/2010-102/2S | May, 12 | swine | BK1LF1 | LC149657 |
| O/JPN/2010-103/1S | May, 12 | swine | IB1LF1 | LC149658 |
| O/JPN/2010-104/2S | May, 12 | cattle | BK1LF1 | LC149659 |
| O/JPN/2010-105/2S | May, 12 | cattle | BK1LF1 | LC149660 |
| O/JPN/2010-106/4S | May, 12 | cattle | BK1LF1 | LC149661 |
| O/JPN/2010-107/5S | May, 13 | cattle | BK1LF1 | LC149662 |
| O/JPN/2010-108/5S | May, 13 | cattle | BK1LF1 | LC149663 |
| O/JPN/2010-110/5S | May, 13 | cattle | BK1LF1 | LC149664 |
| O/JPN/2010-111/2S | May, 13 | cattle | BK1LF1 | LC149665 |
| O/JPN/2010-113/2S | May, 13 | cattle | BK1LF1 | LC149666 |
| O/JPN/2010-115/2S | May, 13 | cattle | BK1LF1 | LC149667 |
| O/JPN/2010-118/1S | May, 14 | cattle | BK1LF1 | LC149668 |
| O/JPN/2010-121/3S | May, 14 | cattle | BK1LF1 | LC149669 |
| O/JPN/2010-122/4S | May, 14 | cattle | BK1LF1 | LC149670 |
| O/JPN/2010-123/3S | May, 14 | cattle | BK1LF1 | LC149671 |
| O/JPN/2010-124/5S | May, 14 | cattle | BK1LF1 | LC149672 |
| O/JPN/2010-125/1S | May, 14 | cattle | BK1LF1 | LC149673 |
| O/JPN/2010-126/3S | May, 14 | cattle | BK1LF1 | LC149674 |
| O/JPN/2010-127/1S | May, 15 | cattle | BK1LF1 | LC149675 |
| O/JPN/2010-128/3S | May, 15 | swine | BK1LF1 | LC149676 |
| O/JPN/2010-129/2S | May, 15 | cattle | BK1LF1 | LC149677 |
| O/JPN/2010-133/5S | May, 15 | cattle | BK1LF1 | LC149678 |
| O/JPN/2010-134/2S | May, 15 | swine | BK1LF1 | LC149679 |
| O/JPN/2010-135/1S | May, 15 | cattle | BK1LF1 | LC149680 |
| O/JPN/2010-137/3S | May, 15 | cattle | BK1LF1 | LC149681 |
| O/JPN/2010-138/3S | May, 15 | cattle | BK1LF1 | LC149682 |
| O/JPN/2010-140/1S | May, 15 | cattle | BK1LF1 | LC149683 |
| O/JPN/2010-141/3S | May, 15 | swine | BK1LF1 | LC149684 |

**Table 6.** (Continued)

| Isolate | Date of sampling | Species | Passage history | GenBank accession no. |
|---|---|---|---|---|
| O/JPN/2010-142/1S | May, 15 | cattle | BK1LF1 | LC149685 |
| O/JPN/2010-144/2S | May, 16 | cattle | IB1LF1 | LC149686 |
| O/JPN/2010-145/5S | May, 16 | cattle | BK1LF1 | LC149687 |
| O/JPN/2010-146/3S | May, 16 | swine | IB1LF1 | LC149688 |
| O/JPN/2010-148/3S | May, 16 | cattle | BK1LF1 | LC149689 |
| O/JPN/2010-151/1S | May, 16 | swine | BK1LF1 | LC149690 |
| O/JPN/2010-152/2S | May, 16 | swine | BK1LF1 | LC149691 |
| O/JPN/2010-157/3S | May, 16 | swine | BK1LF1 | LC149692 |
| O/JPN/2010-159/3S | May, 17 | cattle | BK1LF1 | LC149693 |
| O/JPN/2010-163/3S | May, 17 | cattle | BK1LF1 | LC149694 |
| O/JPN/2010-165/2S | May, 17 | cattle | BK1LF1 | LC149695 |
| O/JPN/2010-168/3S | May, 17 | cattle | BK1LF1 | LC149696 |
| O/JPN/2010-169/1S | May, 17 | swine | BK1LF1 | LC149697 |
| O/JPN/2010-171/2S | May, 17 | cattle | BK1LF1 | LC149698 |
| O/JPN/2010-172/1S | May, 17 | cattle | BK1LF1 | LC149699 |
| O/JPN/2010-173/3S | May, 18 | cattle | BK1LF1 | LC149700 |
| O/JPN/2010-174/1S | May, 18 | swine | BK1LF1 | LC149701 |
| O/JPN/2010-180/3S | May, 18 | swine | BK1LF1 | LC149702 |
| O/JPN/2010-188/2S | May, 19 | cattle | BK1LF1 | LC149703 |
| O/JPN/2010-194/3S | May, 20 | cattle | BK1LF1 | LC149704 |
| O/JPN/2010-195/3S | May, 20 | cattle | BK1LF1 | LC149705 |
| O/JPN/2010-196/3S | May, 20 | cattle | BK1LF1 | LC149706 |
| O/JPN/2010-198/2S | May, 20 | cattle | BK1LF1 | LC149707 |
| O/JPN/2010-199/3S | May, 20 | cattle | BK1LF1 | LC149708 |
| O/JPN/2010-200/3S | May, 20 | cattle | BK1LF1 | LC149709 |
| O/JPN/2010-201/1S | May, 20 | cattle | BK1LF1 | LC149710 |
| O/JPN/2010-202/3S | May, 20 | cattle | BK1LF1 | LC149711 |
| O/JPN/2010-216/3S | May, 21 | cattle | BK1LF1 | LC149712 |
| O/JPN/2010-244/3S | May, 24 | cattle | BK1LF1 | LC149713 |
| O/JPN/2010-247/3S | May, 24 | cattle | IB1LF1 | LC149714 |
| O/JPN/2010-253/2S | May, 24 | cattle | IB1LF1 | LC149715 |
| O/JPN/2010-290/1S | May, 29 | cattle | BK1 | LC149716 |
| O/JPN/2010-324/3 | June, 01 | cattle | BK1ZR1 | LC149717 |
| O/JPN/2010-351/1S | June, 09 | cattle | BK1LF1 | LC149718 |

**Table 6.** (Continued)

| Isolate | Date of sampling | Species | Passage history | GenBank accession no. |
|---|---|---|---|---|
| O/JPN/2010-354/3 | June, 10 | cattle | BK1LF1 | LC149719 |
| O/JPN/2010-362/3 | June, 11 | swine | BK1LF1 | LC149720 |

[a] BK, primary bovine kidney cell; LF, LFBK-αvβ6 cell; IB, IB-RS-2 cell; ZR, ZZ-R 127 cell

**Table 7.** Variability of each protein-coding region of virus isolates obtained from the 2010 epidemic in Japan

| Genome region[a] | | No. of positions aligned | Average identity rate (%) | | Invariant rate (%) |
|---|---|---|---|---|---|
| L-fragment | nt | 7,668 | 99.83 | (99.56-99.98) | 95.25 |
| L | nt | 603 | 99.81 | (99.00-100) | 93.70 |
| | aa | 201 | 99.67 | (97.51-100) | 89.55 |
| VP4 | nt | 255 | 99.92 | (99.21-100) | 96.08 |
| | aa | 85 | 99.93 | (97.64-100) | 96.47 |
| VP2 | nt | 654 | 99.69 | (98.77-100) | 93.27 |
| | aa | 218 | 99.46 | (98.16-100) | 93.12 |
| VP3 | nt | 660 | 99.86 | (98.93-100) | 95.00 |
| | aa | 220 | 99.82 | (97.72-100) | 92.73 |
| VP1 | nt | 639 | 99.86 | (99.06-100) | 94.05 |
| | aa | 213 | 99.76 | (97.65-100) | 89.20 |
| 2A | nt | 48 | 99.76 | (95.83-100) | 89.58 |
| | aa | 16 | 99.88 | (93.75-100) | 93.75 |
| 2B | nt | 462 | 99.77 | (98.05-100) | 94.59 |
| | aa | 154 | 99.75 | (95.45-100) | 91.56 |
| 2C | nt | 954 | 99.90 | (99.47-100) | 96.96 |
| | aa | 318 | 99.93 | (99.05-100) | 96.54 |
| 3A | nt | 459 | 99.84 | (98.91-100) | 94.12 |
| | aa | 153 | 99.76 | (98.03-100) | 92.81 |
| 3B | nt | 213 | 99.54 | (98.12-100) | 93.90 |
| | aa | 71 | 99.60 | (97.18-100) | 91.55 |
| 3C | nt | 639 | 99.90 | (99.21-100) | 97.34 |
| | aa | 213 | 99.74 | (98.23-100) | 95.77 |
| 3D | nt | 1,413 | 99.87 | (99.36-100) | 96.46 |
| | aa | 471 | 99.87 | (98.93-100) | 95.97 |

[a] nt, nucleotide; aa, amino acid

**Table 8.** Variable amino acid positions of FMDVs obtained from the 2010 epidemic in Japan

| Genome region | aa no.[a] | aa | Proportion (%) | No. of viruses |
|---|---|---|---|---|
| L | 17 | Ile | 91.4 | 95 |
| | | Thr | 6.7 | 7 |
| | | Lys | 1.0 | 1 |
| | | Met | 1.0 | 1 |
| VP2 | 78 | Cys | 92.3 | 96 |
| | | Tyr | 4.8 | 5 |
| | | Tyr/Cys | 2.9 | 3 |
| | 79 | Tyr | 80.8 | 84 |
| | | His | 9.6 | 10 |
| | | Cys | 5.8 | 6 |
| | | Tyr/His | 3.8 | 4 |
| VP2 | 132 | Ile | 92.3 | 96 |
| | | Thr | 2.9 | 3 |
| | | Ile/Thr | 4.8 | 5 |
| VP2 | 182 | Met | 84.6 | 88 |
| | | Thr | 6.7 | 7 |
| | | Val | 4.8 | 5 |
| | | Met/Thr | 3.8 | 4 |
| 3C | 63 | Ile | 82.7 | 86 |
| | | Thr | 17.3 | 18 |
| 3D | 158 | Ala | 92.3 | 96 |
| | | Val | 7.7 | 8 |

[a] aa, amino acid

(a)

**Fig. 4. Phylogenetic analysis of FMDV isolates using L-fragment gene.**

(a) 7,668 nucleotide bases of the whole L-fragment of FMDVs were used for ML phylogenetic analysis based on the Tamura-Nei model. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at each node indicate the confidence level in bootstrap analysis with 1,000 replications. (b) 7,668 nucleotide bases of the whole L-fragment of FMDVs were used for MCMC method using the BEAST program based on the Tamura-Nei model. Isolation dates were used to calibrate the molecular clock. MCMC chains were run for 10 million states, sampled every 1,000 states. One-hundred and four virus isolates obtained in the present study were colored gray.

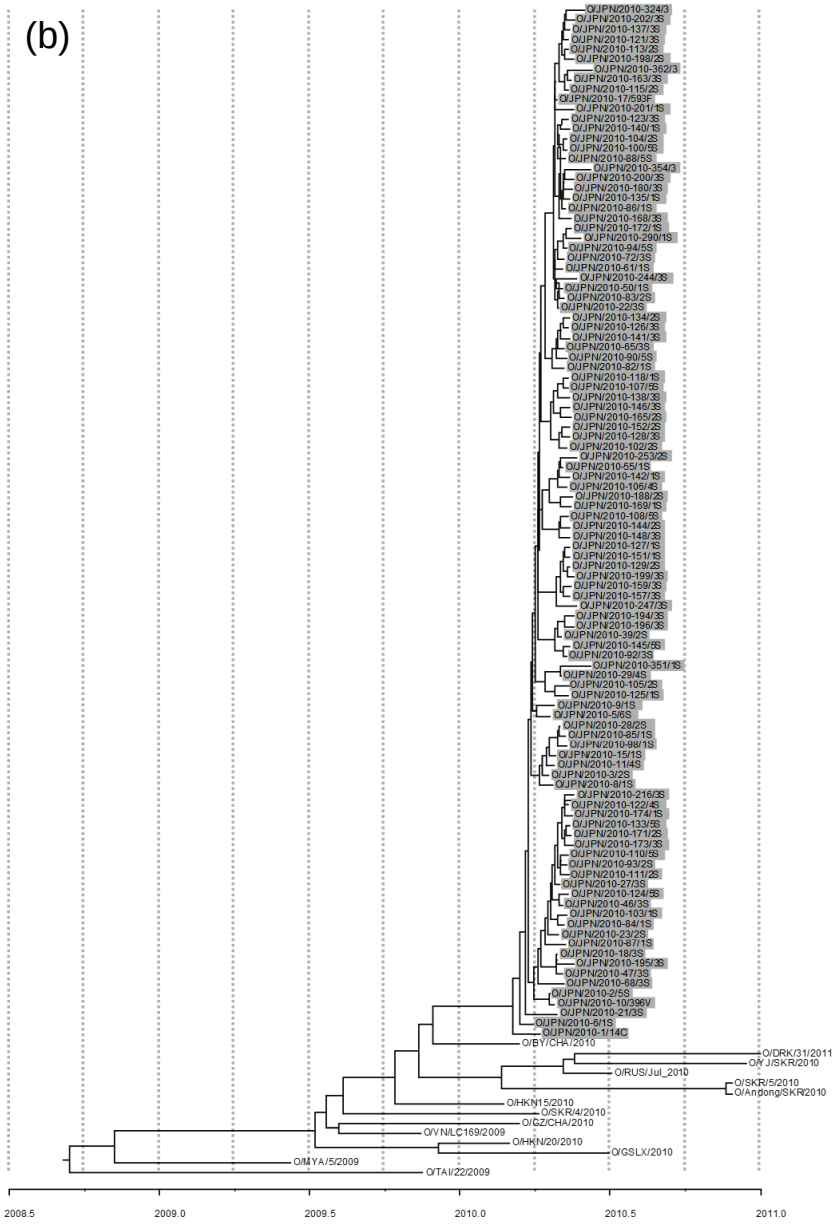


**Fig. 4. Continued**

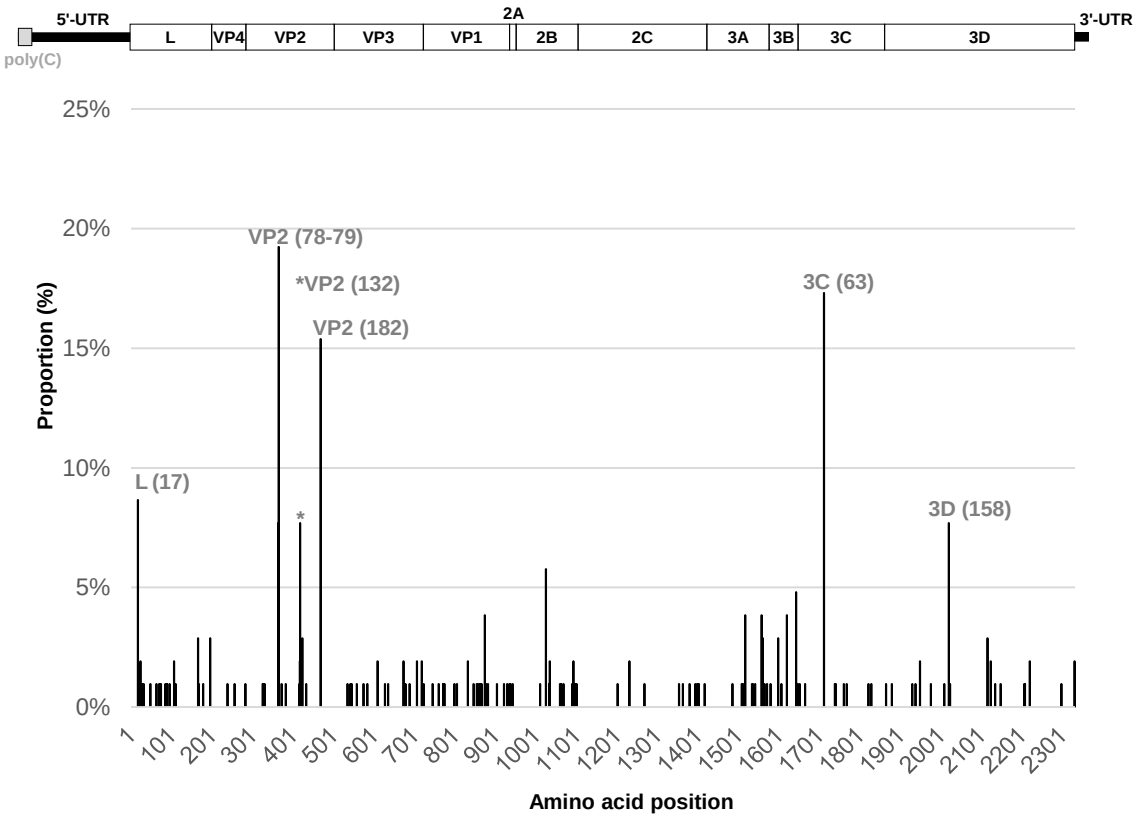


**Fig. 5. Variable amino acid positions of FMDV isolates obtained from the 2010 epidemic in Japan.**

Proportion of samples with substitution of amino acids on each amino acid positions of 104 virus isolates compared with the consensus sequence of them were calculated. Six positions at which proportion of samples with substitution were more than 7.7% (8 of 104 virus isolates) were indicated with protein coding regions belonging and amino acid number inside the parentheses.

# Chapter III

## 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

# Introduction

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 (17, 18). In the 2000 epidemic, outbreak was limited to four cattle farms and eradicated by slaughter of 740 cattle. In contrast, the FMD outbreak in 2010 spread to 292 farms. The differences in infectivity and pathogenicity of the viruses were suspected to contribute to the relative severity of the 2010 outbreak compared to the one in 2000; however, the molecular mechanisms underlying the pathogenicity of O/JPN/2000 and O/JPN/2010 are not well understood. In 2000, an FMD outbreak caused by the virus of serotype O, ME-SA/PanAsia occurred in Japan (17). According to phylogenetic analysis of VP1 sequence, this strain was firstly confirmed in India, 1990 and spread west- and eastward including South Korea, China, Taiwan, Mongolia and Russia (7). On the other hand, in 2010, FMDV of serotype O, SEA/Mya-98 was introduced into Japan. This strain has been endemic in mainland Southeast Asia and introduced into Japan via the neighboring country as described in Chapter II. Viruses of these lineages, namely O/ME-SA/PanAsia and O/SEA/Mya-98, have been circulating in the Southeast Asia and caused many sporadic outbreaks in East Asia in recent years (48). Therefore, studies on the molecular characterization of these strains which caused extensive spread in the world are valuable to elucidate pathogenicity of FMD.

To date, infectious cDNA clones of FMDV have been constructed for the purposes of understanding viral replication and pathogenicity at the molecular level as well as for vaccine development (49-56). Recombinant and site-directed mutants have been used for genomic studies of FMDV (9, 57-63). Here, in Chapter III, a full-length infectious cDNA clone of O/JPN/2010 was constructed and the recovered virus was compared to its parental strain by *in vitro* characterization and experimental infection of pigs.

# Materials and Methods

### *Cells and viruses*

BK, African green monkey kidney (Cos-7) and IB-RS-2 cells were grown in MEM (Nissui Pharmaceutical), while LFPK-αvβ6 cells (39, 40) and ZZ-R 127 cells (27) were grown in DMEM (Gibco-BRL) supplemented with fetal bovine serum. The cells were maintained at 37°C in a 5% $CO_2$ atmosphere. The FMDV used in this experiment (O/JPN/2010-290/1E) was isolated from the epithelial tissue of infected cattle, which was confirmed as the 235th case of 292 in total during the 2010 epidemic in Japan.

### *Full-length FMDV cDNA construction*

Viral RNA was extracted using a High Pure Viral RNA Kit (Roche Diagnostics). First-strand cDNA synthesis was performed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics) and random hexamer primers. PCR amplifications were performed using Platinum Taq Polymerase High Fidelity (Life Technologies) and an Applied Biosystems (ABI) GeneAmp PCR System 9700 (Life Technologies). The primers used for PCR were designed based on the sequence of the O/JPN/2010-290/1E isolate or other reports (Table 9). The 5′- and 3′-sequencing were performed using a SMARTer RACE cDNA Amplification Kit (Clontech, CA, USA) and Universal Primer A Mix in the kit was used as the PCR primer with FM67 (5'-AGCTAAAATGGCTACGCGGTGC-3') and 7678F, respectively. The PCR fragments were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Tokyo, Japan) and inserted into the pGEM-T Easy cloning vector (Promega). To avoid errors in PCR amplification for the construction of full-length cDNA, five clones from each PCR fragment were obtained and nucleotide sequences of the inserts were confirmed using an ABI 3130 genetic analyzer (Life

Technologies) to select a clone which had consensus sequence of these five clones. The full-length cDNA was assembled with five cDNA clones covering the complete genome of O/JPN/2010-290/1E using appropriate restriction enzymes and cloned downstream of the $SV_{40}$ late promoter in the pSVL vector (formerly Pharmacia Biotech, WI, USA) and designated as pSVL-f02.

***Transfection of mammalian cells with cDNA***

Cos-7 and IB-RS-2 were grown in a 12 well culture plate. At 70-90% confluency, 0.1-1,000 ng of pSVL-f02 was transfected using Lipofectamine 3000 (Life Technologies). The culture was incubated at 37°C, in 5% $CO_2$ until a distinct CPE was observed. The supernatants and cells were collected and clarified at 2,000 × g for 10 min after three freeze/thaw cycles. Efficiency of transfection was evaluated by determining viral titers as $TCID_{50}$ in LFPK-αvβ6 cells as described in Chapter I. Furthermore, a time-course analysis was performed for transfected cells. After transfection using 1,000 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 $TCID_{50}$ in LFPK-αvβ6 cells.

***Virus neutralization test (VNT)***

The sera were collected from pigs inoculated with FMDV O/JPN/2010-290/1E isolate (No. 7 and 9) or the recovered virus vSVL-f02 (No. 11 and 12). They were collected at 14 days post inoculation (dpi) except No. 7, which was collected at 10 dpi and showed the highest antibody titer in the liquid-phase blocking ELISA (LPBE) (titers were No.7, 9: 362, No. 11 and 12: 256). Briefly, sera were heat inactivated at 56°C for 30 min, then 50 µL of sera were diluted twofold across 2 rows of a 96-well plate. Next, 50 µl of O/JPN/2010-

290/1E or vSVL-f02 corresponding to $10^2$ $TCID_{50}$ was added into the wells, and the plate was incubated at 37°C in 5% $CO_2$ for 1 hr. One hundred microliters of LFPK-αvβ6 cells were then added to the wells and plates were incubated at 37°C in 5% $CO_2$ for 5 days. Antibody titers were expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells were protected.

***Indirect immunofluorescence test***

The antigenicity of the recovered virus vSVL-f02 was also examined using an indirect immunofluorescence test. IB-RS-2 cell monolayers grown in a 4-well Lab-Tek II chamber slide (Life Technologies) were fixed in acetone one day after virus inoculation. The FMDV-specific monoclonal antibody 1H5, which was raised against the O/JPN/2000 strain and reacts with all seven serotypes (64), was then incubated with the monolayers. 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).

***Examination of plaque size***

The recovered virus vSVL-f02 and the parental 290/1E isolate were inoculated onto IB-RS-2 or LFPK-αvβ6 cell monolayers grown in 6 well plates and incubated at 37°C for 1hr. The monolayers were then overlaid with MEM containing 1% Noble-agar and incubated for 1 day at 37°C in 5% $CO_2$. Monolayers were fixed with 5% formalin and stained with crystal violet to visualize plaques.

***One-step growth***

The recovered virus vSVL-f02 and the parental 290/1E isolate were inoculated onto

LFPK-αvβ6 cell monolayers cultured in 25-$cm^2$ flasks at a multiplicity of infection (MOI) of 2, then the culture supernatant was harvested at subsequent time points and the amount of virus present was determined by plaque assay using LFPK-αvβ6 cells. Ten-fold dilutions of viruses were inoculated onto confluent monolayers of LFPK-αvβ6 cells and incubated at 37°C for 1 hr. Plaques were visualized as described above and the number of plaques was counted in well which 5-50 independent plaques were formed. The plaque forming unit (PFU) was calculated as the product of the reciprocal value of the highest virus dilution and the number of plaques in the dilution.

***Experimental infection of pigs***

To test pathogenicity, vSVL-f02 or 290/1E isolates were inoculated into pigs. Six 2-month-old pigs were numbered for identification and divided into two groups: group 1: No.7-9; group 2: No.10-12. The 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. Virus titers were determined using LFPK-αvβ6 cells as described in Chapter I. Pigs were observed for the appearance of clinical signs daily until 14 dpi. Sera and saliva were collected as described in Chapter I. Nasal swabs were collected from the nasal cavity using a cotton swab (Men-tip, JCB Industry Limited, Tokyo, Japan). The Animal Care and Use Committee of the NIAH approved all animal procedures prior to initiation of this study (authorization number: 14-060). Experimental infections were performed in a high-containment facility at the NIAH.

***Virus isolation and titration***

LFPK-αvβ6 cells were used for virus isolation and titration of collected samples in this study. Virus isolation was performed according to the OIE manual (19). Cell monolayers

in 24-well plates were washed once and were inoculated with 150 µl of the samples for 1 hr at 37°C in 5% $CO_2$, then the monolayers were washed with PBS, fresh media was added and the cultures were incubated for 72 hrs at 37°C in 5% $CO_2$. The monolayers were observed for the appearance of CPE daily. Virus titration was performed as follows: serial tenfold dilutions of the original clinical samples were prepared in tubes, and each dilution was inoculated into four wells of the 24-well plates. Virus titers were calculated by the Reed-Muench method.

***RT-PCR and real-time RT-PCR***

Viral RNAs were extracted from the clinical samples using a High Pure Viral RNA Kit (Roche Diagnostics) according to the manufacturer's instructions. FMDV-specific genes were detected using the FM8-9 primer set previously described in Chapter I. Viral RNA loads were determined by rRT-PCR using primers and a probe targeting 3D region (Table 1), TaqMan Fast Virus 1-Step Master Mix (Life Technologies), and an ABI 7500 Fast Real-Time PCR System (Life Technologies). The number of viral RNA copies was calculated using a standard curve prepared from a positive amplification control containing a portion of the 3D gene of O/JPN/2010 as described in the previous study (29). Viral RNA loads in the sera, saliva and nasal discharges were expressed as $10^x$ copies/ml.

***Antibody detection***

LPBE was performed for detection and titration of antibodies to FMDV according to the manufacturer's instructions. Antibodies against non-structural protein (NSP) of FMDV were detected using the PrioCHECK FMDV NS Antibody ELISA Kit (Life Technologies) according to the manufacturer's instructions.

# Results

### *Construction of a full-length FMDV cDNA*

The genome of FMDV O/JPN/2010-290/1E was determined to be 8,171 nucleotides in length excluding the poly(C) tract between the S- and L-fragments and the poly(A) tail at the 3' end (GenBank accession number LC036265). The five cDNA fragments comprising the genome of FMDV O/JPN/2010-290/1E were amplified and cloned into the pGEM-T Easy vector. For selection, five clones from each PCR fragment were obtained and the clone which had consensus sequence of them were used. These five cDNA clones were ligated to each other and inserted into the pSVL plasmid to create the pSVL-f02 plasmid (Fig. 6). The 5N-2 clone had a poly(C)$_{18}$ tract and the 3R-L4 clone had a poly(A)$_{27}$ tail confirmed by sequencing.

### *Mammalian cell transfection*

In Cos-7 and IB-RS-2 monolayers transfected with 1,000 ng plasmid pSVL-f02 DNA, distinct CPE was observed as soon as 1 day following transfection. The recovered virus, identified as vSVL-f02 was harvested from supernatant and cells at 2 days post-transfection and clarified by centrifugation following three freeze/thaw cycles. 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 the titration of the recovered viruses revealed that at least 100 ng and 10 ng of plasmid were necessary to produce infectious virus in Cos-7 and IB-RS-2 cells, respectively (Table 10A). In the time-course analysis, the highest titers were detected at 2 and 1 day(s) post transfection in Cos-7 and IB-RS-2 cells, respectively (Table 10B). The infectious virus

from Cos-7 cells was passaged three times on the fetal goat tongue cell line ZZ-R 127, then subsequently on BHK-21 and ZZ-R 127 cells to obtain a high titer viral sample ($10^{7.3}$ $TCID_{50}$/0.1mL) that was used for subsequent studies. Virus recovered from IB-RS-2 cells was not used in further experiments, as the IB-RS-2 cells were found to be contaminated with bovine viral diarrhea virus.

***Characterization of recovered virus in vitro***

The FMDV O/JPN/2010-290/1E isolate and recovered virus vSVL-f02 were neutralized by sera collected from pigs inoculated each virus and there were no remarkable differences between the antibody titers (Table 11). In an indirect immunofluorescence test using the FMDV-specific monoclonal antibody 1H5, a characteristic cytoplasmic staining was confirmed on cells infected with vSVL-f02, whereas no staining on uninfected cells were observed (Fig. 7). On the plaque growth assay of parental virus 290/1E and the recovered virus vSVL-f02 IB-RS-2 and LFPK-αvβ6 cell monolayers, obvious difference was not confirmed (Fig. 8). Their plaque sizes on IB-RS-2 were not clonal may be due to the feature of this cell line lacking contact inhibition. Growth curves of both viruses reached a plateau at 12 hours post inoculation and there were no significant differences in virus titers at each time points of the growth step calculated by Student's t-test (Fig. 9). Therefore, the characteristics of the recovered virus *in vitro* were identical to those of the parental strain.

***Pathogenicity in pigs***

All pigs infected with either the parental virus or the recovered virus vSVL-f02 developed vesicles by 1 or 2 dpi on their feet, lips, and tongues (Table 12). 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. 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 from the sera (1-8 dpi), saliva (2-14 dpi) and nasal swabs (1-9 dpi) collected from vSVL-f02 inoculated pigs. Antibodies were detected by LPBE from 5 or 6 dpi in both groups and the NSP-specific antibodies were detected from 7 or 8 dpi, and 6-8 dpi in groups 1 and 2, respectively. Viral titers measured in the sera, saliva, and nasal swabs collected from pigs in group 1 were $10^{2.1}$-$10^{6.1}$ $TCID_{50}$/ml, $10^{2.6}$-$10^{6.3}$ $TCID_{50}$/ml and $10^{2.6}$-$10^{6.6}$ $TCID_{50}$/ml, respectively, while the viral RNA loads were $10^{6.6}$-$10^{9.8}$ copies/ml, $10^{7.5}$-$10^{10.6}$ copies/ml and $10^{7.7}$-$10^{10.2}$ copies/ml (data not shown). Viral titers measured in sera, saliva, and nasal swabs collected from pigs in group 2 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, while the viral RNA loads were $10^{6.9}$-$10^{9.5}$ copies/ml, $10^{7.7}$-$10^{10.1}$ copies/ml and $10^{7.4}$-$10^{10.3}$ copies/ml (data not shown). There were no remarkable differences in clinical signs, virus isolation and titers, viral RNA loads and antibody responses observed between groups 1 and 2.

## Discussion

Plasmid pSVL-f02 containing a full-length cDNA of FMDV O/JPN/2010-290/1E isolate was constructed and the vSVL-f02 virus was recovered following transfection of mammalian cells. The *in vitro* characteristics of vSVL-f02, including its antigenicity in the VNT, indirect immunofluorescence, and plaque size and one-step growth, were identical to those of the parental 290/1E isolate. In the animal experiments, pigs inoculated with either the FMDV O/JPN/2010-290/1E isolate or the vSVL-f02 isolate developed vesicles on their feet, lips and tongues beginning at 1 or 2 dpi. In addition, there were no significant differences between groups 1 and 2 in the dynamics of viral loads in serum, saliva, and nasal swabs or in the development of anti-FMDV and anti-NSP antibodies. These data demonstrate that the *in vitro* and *in vivo* characteristics of the virus recovered from pSVL-f02 transfected cells were consistent with those of parental virus. Therefore, it was concluded that a full-length cDNA of FMDV O/JPN/2010-290/1E was successfully cloned and constructed.

The pSVL-f02, which is based on pSVL, harbors a full-length FMDV cDNA within the SV40 VP1 translational unit and carries the SV40 origin of replication. Therefore, the replication efficiency of this plasmid should theoretically be greatly increased in Cos-7 cells (which express the SV40 large T antigen) and result in high levels of protein expression following DNA transfection (65). In this study, contrary to expectations, transfection efficiency did not increase in Cos-7 cells compared to IB-RS-2 cells. The reason for this is unknown; however, it was probably due to the difference in susceptibility to infection with FMDV between Cos-7 and IB-RS-2 cells.

The studies reported herein have demonstrated that the virus recovered from a full-length cDNA of FMDV O/JPN/2010-290/1E isolate retained all of the biological

characteristics of the parent isolate. This FMDV infectious cDNA is therefore a valuable tool with which to analyze the determinants of pathogenicity of FMDV, as well to conduct further studies to understand the mechanisms of virus replication.

## Summary

A full-length infectious cDNA clone of the genome of a FMDV isolated from the 2010 epidemic in Japan was constructed and designated pSVL-f02. Transfection of Cos-7 or IB-RS-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 FMDV.

**Table 9.** Oligonucleotide primers used for construction of a full-length genomic cDNA clone of FMDV O/JPN/2010-290/1E isolate

| Primer | Sequence | Fragment | Nucleotide position[a] |
|---|---|---|---|
| F1 | TTGAAAGGGGGCGCTAGGGT | 5'-UTR | 1-20 (+) |
| OIE primer 2[b] | CCAGTCCCCTTCTCAGATC | 5'-UTR | 990-1008 (–) |
| OIE primer 1[c] | GCCTGGTCTTTCCAGGTCT | SP | 683-701 (+) |
| 2B58[d] | GACATGTCCTCCTGCATCTG | SP | 4014-4033 (–) |
| 1D196 | CTCCGCACTGCCACTTACTATT | NSP | 3465-3486 (+) |
| FM9 | CCTTTGTCGCTTTTGTCAGCTGG | NSP | 7779-7801 (–) |
| 6680F | GGGTTGATCGTAGACACCAGAGATGT | 3D | 6684-6709 (+) |
| 8092R | TTATGCGCCACCGCACACG | 3D | 8078-8096 (–) |
| 7678F | GATCTCCTACGGAGACGACATCGTGGTT | 3'-UTR | 7682-7709 (+) |
| UPM[e] | CTAATACGACTCACTATAGGGC | 3'-UTR | in 3'RACE kit |

SP, structural protein; NSP, non-structural protein

[a] Nucleotide position corresponds to the nucleotide sequence of FMDV O/JPN/2010-290/1E (LC036265).

[b,c] Both primers were described in OIE Manual (19).

[d] previously described as NK61 (25)

[e] Universal Primer A Mix (UPM) in SMARTer RACE cDNA Amplification Kit (Clontech, CA, USA)

**Table 10A.** Virus titers in supernatants of transfected Cos-7 and IB-RS-2 cells with various amounts of pSVL-f02 DNAs

| Cells | DNA (ng) | Virus titers in supernatants ($log_{10}$ $TCID_{50}$/0.1ml) |
|---|---|---|
| Cos-7 | 1,000 | 1.8 |
| | 100 | 0.6 |
| | 10 | – |
| | 1 | – |
| | 0.1 | – |
| IB-RS-2 | 1,000 | 3.0 |
| | 100 | 1.7 |
| | 10 | 1.0 |
| | 1 | – |
| | 0.1 | – |

–, Not detectable

**Table 10B.** Time-course of virus titers after transfection of Cos-7 and IB-RS-2 cells with 1,000 ng of pSVL-f02

| Cells | Days post transfection | Virus titers ($log_{10}$ $TCID_{50}$/0.1ml) | |
|---|---|---|---|
| | | Sup | Sup + cell |
| Cos-7 | 1 | 1.4 | 1.7 |
| | 2 | 1.8 | 2.3 |
| | 3 | 1.2 | 2.1 |
| IB-RS-2 | 1 | 2.0 | 2.6 |
| | 2 | 0.6 | 0.8 |
| | 3 | – | – |

Sup, Supernatant; –, Not detectable

**Table 11.** Comparison of antigenicity of 290/1E isolate and vSVL-f02

| | Sera from pigs infected with | | | |
|---|---|---|---|---|
| | 290/1E | | vSVL-f02 | |
| Virus | 1[a] | 3 | 5 | 6 |
| 290/1E | 90[b] | 181 | 32 | 128 |
| vSVL-f02 | 181 | 256 | 64 | 181 |

[a] Pig No.
[b] Sera were diluted twofold. Neutralization titers were expressed as the final dilution of sera where 50% of wells are protected. Homologous titers were underlined.

**Table 12.** Time course of infection in pigs inoculated with 290/1E isolate and recovered virus vSVL-f02

| Pig No. | Virus | Clinical sample | Virus isolation/genetic detection and antibody detection at each day post infection[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 13/14[b] |
| 1 | 290/1E | Serum | –/– | +/+ | +/+ | +/+ | –/– | –/– | –/– | –/– | –/– | –/– | –/– | –/– |
| | | Saliva | –/– | +/+ | +/+ | +/+ | +/+ | –/+ | –/+ | –/+ | –/+ | –/+ | –/+ | –/– |
| | | Nasal swab | –/– | –/– | +/+ | +/+ | –/+ | –/+ | –/+ | –/– | –/– | –/– | –/+ | –/– |
| | | LPBE | <32 | <32 | <32 | <32 | <32 | 45 | 90 | 181 | 256 | 181 | 362 | 181 |
| | | NSP | – | – | – | – | – | – | – | + | + | + | + | + |
| 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 | – | – | – | – | – | – | – | – | + | + | + | + |
| 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 from Sera, saliva and nasal swabs were indicated. Antibodies against FMDV were detected and titrated by LPBE and PrioCHECK FMDV NS Antibody ELISA Kit.
[b] Samples from pig No. 1-3 and 4-6 were collected at 13 and 14 dpi, respectively.

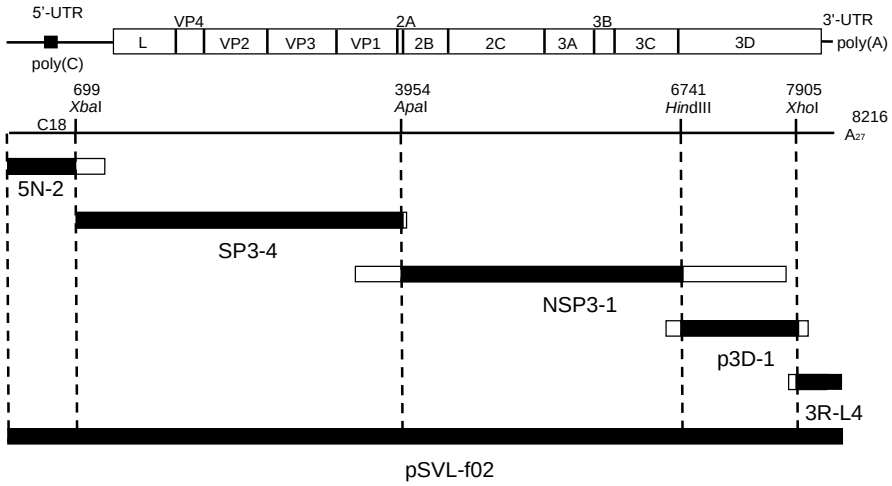


**Fig. 6. Construction of full-length cDNA clone of the FMDV O/JPN/2010-290/1E isolate.**

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.

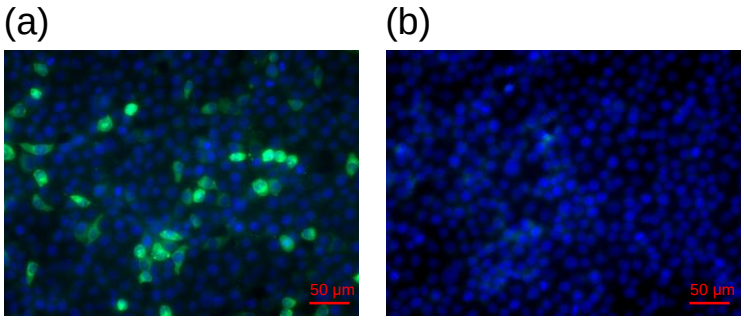


**Fig. 7. Indirect immunofluorescence test of IB-RS-2 cells infected with vSVL-f02.**

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.

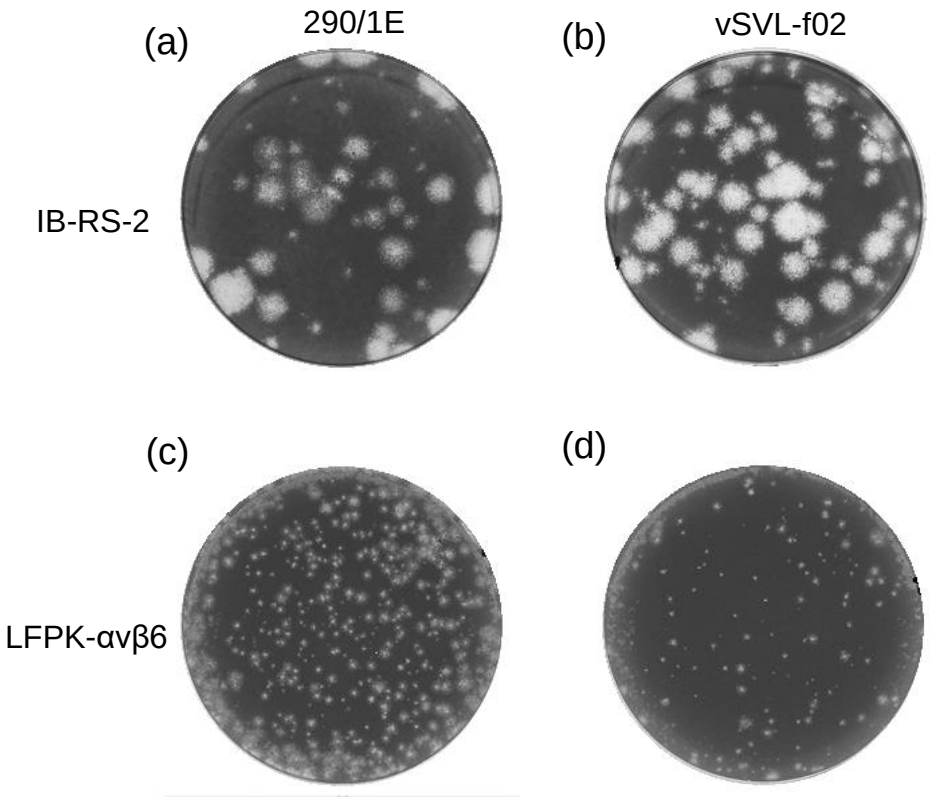


**Fig. 8. Comparison of plaque size of 290/1E isolate and vSVL-f02.**
The 290/1E isolate was inoculated onto IB-RS-2 (a) or LFPK-αvβ6 (c) monolayer cells, respectively. vSVL-f02 was also inoculated onto IB-RS-2 (b) or LFPK-αvβ6 (d) monolayer cells, respectively. The cells were fixed 1 day after the inoculation and stained with crystal violet.

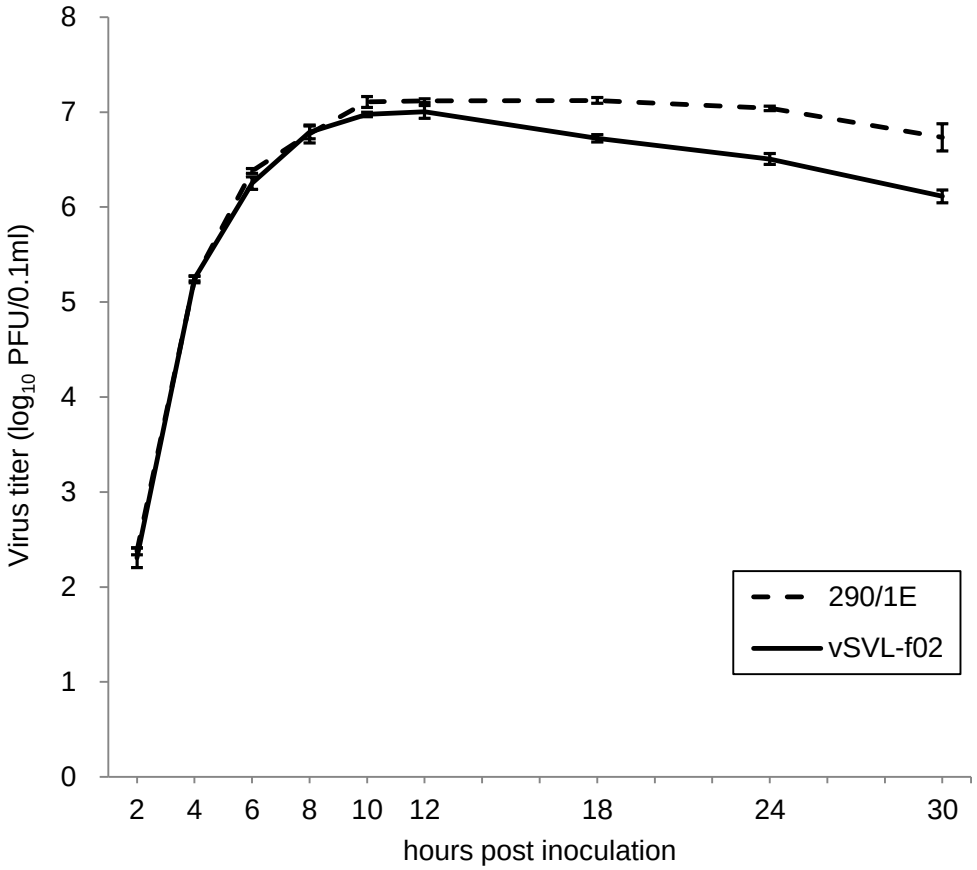


**Fig. 9. One-step growth curves of 290/1E isolate and vSVL-f02.**
The LFPK-αvβ6 cell monolayers were inoculated with each virus at a MOI of 2 and incubated at 37˚C. Samples of supernatant were collected at the indicated times and viral infectivity was determined using a standard plaque assay.

# Chapter IV

# Genetic determinants of pathogenicity between two foot-and-mouth disease virus isolates which caused outbreaks of differing severity

# Introduction

FMD outbreaks in Japan in 2000 and 2010 suggest that the differences in patogenicity of FMDV strains in the susceptible animals could be one factor which lead the differences in severity among these outbreaks. The previous studies have reported the relation of the pathogenicity in hosts and virus growth in cell culture, and responsible genetic factors including amino acid substitution on the capsid proteins, deletion of $L^{pro}$ or part of 3A, and modulation of IRES-3'UTR (12-16). To date, however, few studies have described the genes responsible for its pathogenicity among multiple topotypes or the molecular mechanisms underlying these differences.

In Chapter II, the genome variability of FMDV O/JPN/2010 strains, which caused severe economic damage to livestock industries in Japan, over an epidemic was elucidated. In Chapter III, a full-length infectious cDNA clone of O/JPN/2010 was constructed. The recovered virus was confirmed to retain the infectivity in cell lines and pathogenicity in pigs of their parental strain.

Here, in Chapter IV, the infectivity of two virus strains isolated from the two outbreaks and having different topotypes, O/JPN/2000 and O/JPN/2010, were compared in cattle. Their viral growth in cell culture, and virulence in suckling mice were also compared. In addition, the genes responsible for the difference in infectivity were evaluated using genetic recombinants between the two strains.

# Materials and Methods

***Cells and viruses***

BK, IB-RS-2, BHK-21, Cos-7 and CPK cells were grown in MEM (Nissui Pharmaceutical). ZZ-R 127 (27) and LFPK-αvβ6 cells (39, 40) were grown in DMEM (Gibco-BRL) supplemented with fetal bovine serum. The cells were maintained at 37°C in a 5% $CO_2$ atmosphere. Virus isolation was performed according to the OIE Terrestrial Manual (19). During the outbreak in 2000 and 2010, cells derived from both bovine kidney and porcine kidney were used for the isolation of FMDV from clinical samples because of their high susceptibilities. Cos-7 cells are efficiently transfected with pSVL, thus used for DNA transfection. LFPK-αvβ6 cell was used for virus titration and neutralization test because of its high sensitivity to FMDV and efficient growth of itself.

The virus strain O/JPN/2010-290/1E (GenBank: LC036265) was isolated from an epithelial tissue of cattle in Japan using CPK cells and passaged three times in CPK cells as previously described in Chapter III. O/JPN/2000 (GenBank: AB079061/062) were isolated from a probang material of cattle in Japan using BK cells (17) and passaged two times in BK cells and two times in LFPK-αvβ6 cells. As with the previous study (12), O/JPN/2000 after few passages in BK or BHK-21 cell lines shows small plaque and avirulent pathogenicity in suckling mice due to two substitutions on the 133rd amino acid in VP2 and the 56th amino acid in VP3. On the other hand, LFPK-αvβ6 cell stably express both the αV and β6 bovine integrin subunits which is a principal receptor for FMDV in host cells. Therefore, in this study, viral stock of O/JPN/2000 strain was prepared using LFPK-αvβ6 cell to keep its infectivity. All stock viruses were stored at –80°C.

***Experimental infection of O/JPN/2000 in Holstein cows***

Two six-month-old Holstein cows were inoculated subepidermo-lingually with 1 ml of $10^6$ $TCID_{50}$ (titrated using IB-RS-2 cells) of the FMDV O/JPN/2000 as described previously (32). Two additional Holstein cattle of the same age were housed with them at 0 dpi. This experimental infection was performed in cubicles of approximately 14 $m^2$ in a high containment facility at the NIAH. The cubicles were kept at 25°C and provided 10 to 15 air changes per hour during the experimental period. Clinical signs, virus excretion and antibody responses of the infected animals were observed for approximately 2 weeks. Esophageal-pharyngeal fluid was collected using a probang cup. Collection of clinical samples except for the esophageal-pharyngeal fluid was performed daily until 10 dpi, and at 2-day intervals thereafter. The esophageal-pharyngeal fluids were collected at 0, 10, 12 and 14 or 15 dpi. Clinical signs were scored as follows: each foot bearing a lesion, 1 point; lesions in or around the mouth, 1 point; and lameness, dullness or fever (40°C or more), 1 point. Accordingly, maximum score per animal was 6.

***Virus isolation and titration***

The LFPK-αvβ6 cells were prepared using DMEM supplemented with 10% FBS in 24-well plates at 2 days before virus isolation. Ten-fold dilutions of the clinical samples were serially prepared in tubes in order to determine the virus titers in the samples. After the cells were washed once, a 100 μl volume of each dilution of the clinical samples was transferred to 4 wells of the 24-well plates and incubated at 37°C for 1 hr. The cells were washed again and added to the DMEM supplemented with 10% FBS. The cells were incubated at 37°C for 72 hr in 5% $CO_2$ and observed microscopically for the appearance of a CPE. Virus isolation and titration were performed on the day when each clinical sample was obtained in order to minimize any decrease in virus titers during chilled storage or due

to the freezing and thawing processes. Virus titers were calculated by the Reed-Muench method.

***Virus neutralization test***

VNT was performed using LFPK-αvβ6 cells as previously described in Chapter III. O/JPN/2000 were used as antigens in the VNT in order to determine antibody responses to the virus in the infected animals.

***Viral growth in cell culture***

The O/JPN/2000 and O/JPN/2010-290/1E strains were inoculated onto BK or LFPK-αvβ6 cell monolayers cultured in 12-well plates at a MOI of 0.1. The culture supernatant was then harvested at subsequent time points and the amount of virus present was determined by virus titration using LFPK-αvβ6 cells. To identify plaque morphology of the two strains, they were inoculated onto IB-RS-2 or ZZ-R 127 cell monolayers grown in 6-well plates and incubated at 37°C for 1 hr. The monolayers were then overlaid with MEM containing 0.8% Noble-agar and incubated for 1 day at 37°C in 5% $CO_2$. Monolayers were fixed with 5% formalin and stained with crystal violet to visualize plaques.

***Nucleotide sequencing***

Viral RNA was extracted from the supernatant of infected cells using a High Pure Viral RNA Kit (Roche Diagnostics) and the L-fragment gene, of approximately 7.7 kbs, was amplified by PCR using four pairs of FMDV-specific primers. The nucleotide sequences were analyzed using the Ion PGM system as previously described in Chapter II. The genomes were annotated using the GENETYX software (GENETYX) with the O/JPN/2010-290/1E (GenBank: LC036265) as the reference sequence. The locations of

the amino acid differences found in the VP1 and 3D between O/JPN/2000 and O/JPN/2010 in the three-dimensional structure were indicated using MOE software (Chemical Computing Group, Quebec, Canada). The amino acid differences were plotted on the three-dimensional structure of VP1 or 3D obtained from the Protein Data Bank (PDB; accession numbers 5NER or 4WZM, respectively).

***Infection of suckling mice***

Animal experiments using suckling mice were performed according to the method described by Platt (66). Two-to-five-day-old BALB/c suckling mice were inoculated intraperitoneally with 100 μl of each serially diluted virus with DMEM. Suckling mice were observed for a week after inoculation. The 50% lethal dose ($LD_{50}$) was calculated using the Reed-Muench method.

***Cloning of virus genes and rescue of chimeric viruses***

The 5'-UTR to L gene, IRES, the first half and the second half of P1, P2, P3, VP1, 3A-B 3C and 3D genes of O/JPN/2000 were amplified by PCR using KOD-Plus-Neo (ToYoBo, Osaka, Japan) and the primers described in Table 15. The 5'-UTR to L gene, IRES, the first half and the second half of P1, P2, P3 genes were then ligated with the vectors using appropriate restriction enzymes described in Fig. 12 and a TaKaRa DNA Ligation kit Ver. 2.1. pSVL-f02, which was constructed in the present study, was subjected to PCR to amplify insertion vectors, except for VP1, 3A-B 3C and 3D genes, which were conducted using a KOD-Plus Mutagenesis Kit and the primers described in Table 15 and recombined using an In-Fusion HD Cloning Kit (TaKaRa). DNA transfection was performed using Lipofectamine 3000 (Life Technologies) according to the manufacturer's

protocol. Briefly, 2.5 ng of each plasmid was transfected into Cos-7 cells grown to 70-90% confluence in each well in a 12-well culture plate. The culture was incubated at 37°C in 5% $CO_2$ for three days. The supernatants and cells were collected and clarified by low-speed centrifugation at 5,000 × g for 10 min after two freeze/thaw cycles. The recovered virus from cells transfected with pSVL-f02 was named vSVL-f02, and other chimeric viruses were named as in Table 15. For chimeric viruses, passage history was aligned with parental virus vSVL-f02 as previously described in Chapter III. Namely, the infectious virus from Cos-7 cells was passaged three times on ZZ-R 127, then subsequently on BHK-21 and ZZ-R 127 cells to obtain a high titer viral sample for subsequent studies. Genome sequences of all virus stocks of chimeric viruses were confirmed by the method described above.

***Measurement of mutation frequencies***

Virus stocks of O/JPN/2000 and O/JPN/2010 described above were plaque purified and propagated one time in ZZ-R 127 cells. Viral RNAs were extracted and part of each P1 structural gene was amplified by PCR using the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Life Technologies) with a primer set, 5'-GTGGCATGTAGCGACGGTTA-3'and 5'-CGTGTTTCACTGCCACCTCTAG-3'. The PCR product was cloned using a TOPO TA Cloning Kit (Life Technologies) for sequencing. The sequence data were analyzed using the GENETYX software. For each population, 70 partial P1 sequences of approximately 700 nt per replicate (genome positions 2699-3398 of O/JPN/2010-290/1E) were sequenced. Mutation frequencies per $10^4$ nt were determined as described previously (67).

### *Ethics*

The Animal Care and Use Committee of the NIAH approved all animal procedures prior to the initiation of this study (authorization numbers: 16-001, 17-066 and 18-038). All experimental infections using live viruses were performed in a high-containment facility at the NIAH.

# Results

***Experimental infection with O/JPN/2000 in cattle***

In inoculated cattle 1 and 2, vesicular lesions were initially found at the injection site on the tongue at 1 and 4 dpi, and new lesions began to develop on the feet at 2 and 6 dpi, respectively (Table 13). Lesions on the feet were found at the hindlimbs from 2 or 6 dpi and confirmed at all four limbs on 3 or 7 dpi, respectively. Lameness and/or excess salivation were observed from 1 or 4 dpi, respectively; however, pyrexia was not confirmed. Total clinical scores reached 6 and 5, respectively. From these cattle, virus was isolated from the sera (1-3 or 4-5 dpi), saliva (1-6 dpi), and nasal swabs (2-3 dpi) using LFPK-αvβ6 cells. Antibodies were detected by VNT from 6 or 8 dpi (Fig. 10).

On the other hand, in contact cattle 1 and 2, which were placed in close proximity with the inoculated cattle, although fever was confirmed on 2 and 8 dpi, respectively, no vesicular lesions were confirmed (Table 13). Clinical scores were therefore limited to 1. Viruses were isolated from the sera (7-9 dpc), saliva (8-9 dpc), and nasal swabs (7-10 dpc) of the contact cattle 2. Antibodies were detected by VNT from 11 dpc (Fig. 10). These data indicate that it took 5 days to transmit FMD from inoculated cattle 2 to contact cattle 2.

***Comparison of viral growth in cells of O/JPN/2000 and O/JPN/2010***

O/JPN/2000 and O/JPN/2010 were inoculated onto BK and LFPK-αvβ6 cell monolayers. These were harvested at subsequent time points and the amount of virus present was determined (Fig. 11a-b). Growth curves of both viruses reached a plateau at 6 or 9 hours post inoculation. Virus titers reached $10^{6.1}$ or $10^{6.3}$ $TCID_{50}$/0.1ml at maximum in BK cell, and $10^{6.6}$ or $10^{7.1}$ $TCID_{50}$/0.1ml in LFPK-αvβ6 cell, respectively. Although O/JPN/2010 showed slightly better growth, remarkable difference between them was not

confirmed. Plaques of O/JPN/2000 and O/JPN/2010 on IB-RS-2 and ZZ-R 127 cell monolayers were visualized by staining with crystal violet (Fig. 11c). Even though O/JPN/2000 showed slightly smaller plaques in IB-RS-2, it was confirmed that there was no remarkable difference between plaque sizes of O/JPN/2000 and O/JPN/2010 on ZZ-R 127 cell.

***Comparison of amino acid sequence of O/JPN/2000 and O/JPN/2010***

Genome sequences of O/JPN/2000 and O/JPN/2010 were aligned and the predicted amino acid sequences in each viral protein were compared. Among the 12 proteins which compose the open-reading frame of FMDV, only VP4 showed no difference between the two strains. A total of 106 amino acid differences were confirmed across the whole amino acid sequence (Table 14).

***Construction of recombinant FMDV using the two strains***

Infectious cDNA of O/JPN/2010 was comprehensively recombined to the corresponding positions of O/JPN/2000 (Fig. 13, Table 15). Each recombinant plasmid was transfected into Cos-7 cells and passaged on ZZ-R 127 cells. A total of eight recombinant viruses, with recombined 5'-UTR, IRES, the first half and the second half of P1, VP1, P2, 3A-B and 3D regions between the two strains, showed CPE on ZZ-R 127 cells and were successfully passaged for subsequent studies. Genome sequences of all virus stocks of recombinants were confirmed. Although one nonsynonymous substitution on the 328th nucleotide (from Ser to Pro on the 110th amino acid) in 2B was confirmed in IRES/2000 vSVL-f02, any additional mutations were not observed.

***Pathogenicity of the parental viruses and recombinant FMDV in suckling mice***

Viruses were intraperitoneally inoculated to suckling mice and their survival rate was observed for 7 days. The $LD_{50}$ of O/JPN/2000 and vSVL-f02 were determined to be $10^{2.2}$ and $10^{0.1}$ $TCID_{50}$, respectively, indicating that the recovered virus of infectious cDNA of O/JPN/2010 had higher pathogenicity in suckling mice. Based on this result, the mortality rate of suckling mice inoculated with 10 $TCID_{50}$ of O/JPN/2000 and vSVL-f02 was 0% and 100%, respectively (Fig. 13).

Each recombinant virus was also intraperitoneally inoculated to suckling mice with 10 $TCID_{50}$ and their survival rate was observed (Fig. 13). Chimeric viruses 5'-UTR/2000 vSVL-f02, IRES/2000 vSVL-f02, P1-1st/2000 vSVL-f02, P2/2000 vSVL-f02 and 3A-B/2000 vSVL-f02 showed 100% mortality, as the virulent parental virus, vSVL-f02. This means that the recombination of 5'-UTR, L, VP2 and VP4, P2, 3A and 3B genes did not affect their pathogenicity. In contrast, mortality rates of suckling mice inoculated with 10 $TCID_{50}$ of each of P1-2nd/2000 vSVL-f02, VP1/2000 vSVL-f02 and 3D/2000 vSVL-f02 were 0%. Therefore, VP1 and 3D proteins were individually suspected of being responsible for the pathogenicity of O/JPN/2010 in mice.

***Comparison of amino acid sequence and three-dimensional structure of VP1 and 3D***

Comparison of the VP1 amino acid sequence of O/JPN/2000 and O/JPN/2010 revealed 17 differences (Table 16). Locations of the amino acid difference in the three-dimensional structure of the structural proteins VP1, VP2 and VP3 were predicted using the MOE software (Fig. 14a). Nine of these, including six consecutive amino acid differences, were confirmed near the RGD receptor binding domain.

In the 3D amino acid sequence, 11 differences were identified between the two strains (Table 16). The three-dimensional structure of 3D protein was also analyzed and plotted amino acid differences between the two strains (Fig. 14b). Based on a previous report about the structure of FMDV 3D polymerase (68), three amino acid differences (aa No. 98, 144 and 148) were confirmed on the finger domain; three amino acid differences (aa No. 34, 330 and 425) were confirmed on the palm domain; and one amino acid difference (aa No.469) was confirmed on the thumb domain.

***Comparison of mutation frequencies of O/JPN/2000 and O/JPN/2010***

To determine the mutation frequencies during the propagation in the cell culture of O/JPN/2000 and O/JPN/2010, a 700-bp fragment of the structural protein-coding region from 70 individual clones for each viral population was sequenced and the average number of mutations per $10^4$ nt were calculated. The mutation frequencies of O/JPN/2000 and O/JPN/2010 were 5.21 and 7.98 mutation/$10^4$ nucleotides, respectively.

# Discussion

In this study, Holstein cattle which were intradermally inoculated with O/JPN/2000 unexpectedly showed vesicular development in all four limbs. However, only one of two contact cattle were infected 5 days after virus excretion from inoculated cattle and showed only mild clinical signs (Fig. 10, Table 13). In contrast, in the previous study (32), cattle inoculated with O/JPN/2010 and contact cattle all showed fever, salivation, lameness and vesicular development. Furthermore, the contact cows were confirmed to be infected within only 2 days after virus excretion from the inoculated cattle. Moreover, clinical scores of both inoculated and contacted cattle reached 5 to 6. These data clearly demonstrate that O/JPN/2000 has very low transmissibility and pathogenicity to contact cattle.

Several studies using wild-type strain and their mutants have reported that virus growth in cell culture and pathogenicity in hosts were closely related (12-16). For example, a mutant with a partial deletion in 3A protein did not replicate efficiently in bovine cells *in vitro* and was attenuated in cattle (14, 15). In the present study, in contrast, remarkable difference in viral growth in BK and LFPK-αvβ6 cells were not confirmed between O/JPN/2000 and O/JPN/2010 which belong to different genetic topotypes (Fig. 11). In unweaned mice, on the other hand, which have been widely used as a practical model of FMDV pathogenicity (66, 69, 70), O/JPN/2000 and O/JPN/2010 showed a definite difference in pathogenicity (Fig. 13). The present data support the idea that mortality in infected suckling mice is an effective index for comparing the infectivity of FMDVs, particularly those which belong to different genetic topotypes.

As described above, infectious cDNA of O/JPN/2010 was comprehensively recombined to the corresponding positions of O/JPN/2000, since amino acid differences between the two strains were confirmed all over the genome (Fig. 12, Table 14). A total of

eight recombinant viruses were recovered from transfected cells regardless of the number of amino acid differences in each recombined fragment (Fig. 13). Only a few viruses, such as hepatitis C virus, are known to be flexibly useful for comprehensive fragmental recombination to this extent. This genetic flexibility might be one reason for the enormous genetic variation in FMDV, and allow the generation of recombinants in the field (71). On the other hand, recombinant virus of 3C protein was not recovered. 3C in FMDV plays a role as protease in the viral replication step. Although it has been reported to have "relaxed specificity" which discriminates only weakly in favor of P1-Gln over P1-Glu in contrast to other proteases of picornavirus that strongly favor P1-Gln (72), its adoptability might be restricted in combination with other genetic regions.

Pathogenicity of the parental viruses and recombinant FMDV in suckling mice indicated that VP1 and 3D proteins were individually responsible for the pathogenicity of O/JPN/2010 (Fig. 13). VP1 is the outermost component of the virus particle and is responsible for receptor binding (3, 73). Analysis of the three-dimensional structure of the viral protein showed that 9 of 17 amino acid differences between the two strains were located near the G-H loop (Fig. 14a, Table 16), indicating that the two strains have different selectivity or affinity to host cell receptors. Although one-step growth in the cell monolayers of the two strains were not remarkably different (Fig. 11), in fact, they showed significantly different viral features after serial passages in cells. Namely, O/JPN/2000 and O/JPN/2010 were serially passaged 10 times in BHK-21 cells and inoculated in suckling mice (data not shown). In O/JPN/2000 virus stock at the primary stage, two types of viruses were observed; one shows small plaque and avirulent pathogenicity in suckling mice, whereas another shows large plaque and higher pathogenicity (12). As with the previous study, O/JPN/2000 after the passages showed two substitutions on the 133rd amino acid in VP2 and the 56th amino acid in VP3, which is known as a heparin sulfate binding site and

which influences plaque size and pathogenicity in cattle (74), and significantly decreased mortality in suckling mice ($LD_{50}$, $>10^{3.0}$ $TCID_{50}$). On the other hand, no nonsynonymous substitution or change in mortality in suckling mice was confirmed in serially passaged O/JPN/2010. This data also supports the hypothesis that the two strains have different selectivity to host cell receptors.

The finding that the capsid coding sequences are determinants of FMDV pathogenicity is consistent with a previous study using inter-serotypically recombined chimeric viruses (75). VP1 has been reported to modulate host immune factors, such as inhibiting type I interferon response in cells by interaction with soluble resistance-related calcium binding protein (76). In addition, previous reports indicate that FMDV infection induces cell death by apoptosis mediated by interaction with the integrin receptor (77, 78). In the previous study, terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL)-positive labeling in pigs inoculated with O/JPN/2000 was weaker than that in pigs inoculated with O/JPN/2010 (79). This finding suggests that the viral function which induces apoptosis differs between O/JPN/2000 and O/JPN/2010. The programmed dead cells are processed into an apoptotic small body which is phagocytosed by macrophages. Although FMDV replication in macrophages has not been confirmed, the majority of macrophages carried infectious virus for 10-24 hr. Such macrophages would play a role in the transport of infectious FMDV to different sites in the body, where it could be released to infect other cells for replication (80, 81). Therefore, the selectivity or affinity for receptors of the virus is probably related to its infectivity in cattle. Further study of these protein functions would help elucidate the mechanism of pathogenicity of FMDV.

3D protein of FMDV performs as an RNA polymerase and has a right-hand structure composed of finger, palm, and thumb domains (68). According to a three-dimensional structure analysis of the protein, three, three and one amino acid differences were found on

the finger, palm and thumb domains, respectively (Fig. 14b). In addition, 6 of 11 amino acid differences were confirmed in the region suggested to be responsible for protein-protein interaction (68). Among picornaviruses, relationships between the structure and function of coxsackievirus B3 polymerase have been reported (82). Mutations located at the top of the finger domain affect elongation rates, whereas mutations on the palm domain have the greatest effect on mutation frequencies. Interestingly, FMDV with low-fidelity polymerase is reported to be attenuated in the host (83-85). Namely, higher replication fidelity could induce restricted quasispecies diversity and affect the adaptability and pathogenicity of the strain. In their study, mutants which showed 1.51- to 1.88-fold higher replication fidelity exhibited 10- to 100-fold lower virulence in suckling mice, compared to those of wild-type (85). Using this method, the mutation frequencies (mutations per $10^4$ nt) of O/JPN/2000 and O/JPN/2010 in the P1 region (2,699-3,398 nt) were determined. O/JPN/2000 showed approximately 1.53-fold higher fidelity than O/JPN/2010. These data, and the $10^{2.1}$-fold lower virulence of this strain than O/JPN/2010 in suckling mice, demonstrate that replication fidelity is one factor which accounts for the adaptability and virulence of the virus in the host. Further study of the correlations between FMDV polymerase fidelity and pathogenicity will aid the development of live, attenuated FMDV vaccine candidates, as the enhanced replication fidelity promises high stability and safety.

VP1 and 3D sequences of O/JPN/2000 and O/JPN/2010 were aligned and compared with FMDV sequences available in GenBank. Among the 17 VP1 amino acid differences between the two strains, the six consecutive amino acid differences (aa No. 137-142) near the RGD receptor binding domain were specific sequences to each genetic lineage, namely ME-SA/PanAsia and SEA/Mya-98 lineages, respectively, though other amino acid sequences were common in serotype O strains. On the other hand, among the eleven 3D amino acids in Table 16, Asn of 63rd and 262nd, Ser of 330th of O/JPN/2000 were

specifically confirmed in virus strains of ME-SA/PanAsia lineage, whereas those of O/JPN/2010 were common among multiple topotypes. In addition, Ala of 98th was unique to the O/JPN/2000 strain among all FMDV strains in the GenBank, although it is the same attribute with other amino acids confirmed on this position, valine and isoleucine (hydrophobic amino acid). Additional studies are needed to elucidate whether and how these amino acid motifs effect their protein function.

In the present study, it was demonstrated that O/JPN/2000 and O/JPN/2010 had completely different transmissibility in inoculated cattle and virulence in suckling mice, and that this difference was independently due to differences in VP1 and 3D protein. Selectivity of VP1 to receptors and replication fidelity of the polymerase are suspected to be key individual factors in the difference in infectivity and pathogenicity in the host.

## Summary

Efforts to understand the universal mechanism of FMDV infection may be aided by knowledge of the molecular mechanisms which underlie differences in pathogenicity beyond multiple topotypes and serotypes of FMDV. To investigate the molecular mechanisms, the infectivity of O/JPN/2000 and O/JPN/2010, which caused outbreaks of markedly different scales, in cell lines, Holstein cattle and suckling mice were estimated. Viral growth of the two strains in cells was not remarkably different; however, O/JPN/2000 showed apparently low transmissibility in cattle. Mortality rate of suckling mice inoculated intraperitoneally with 10 $TCID_{50}$ of O/JPN/2000 or O/JPN/2010 also differed, at 0% and 100%, respectively. To identify genes responsible for this difference in infectivity, genetic regions of the full-length cDNA of O/JPN/2010 were replaced with corresponding fragments of O/JPN/2000. A total of eight recombinant viruses were successfully recovered and intraperitoneally inoculated to suckling mice. Strikingly, recombinants having either VP1 or 3D derived from O/JPN/2000 showed 0% mortality in suckling mice, whereas other recombinants showed 100% mortality. This finding indicates that VP1, the outermost component of the virus particle, and 3D, an RNA-dependent RNA polymerase, are individually involved in the pathogenicity of O/JPN/2010. Three-dimensional structural analysis of VP1 confirmed that amino acid differences between the two strains were located mainly at the domain interacting with the cellular receptor. On the other hand, the measurement of their mutation frequencies demonstrated that O/JPN/2000 had higher replication fidelity than O/JPN/2010. These findings suggest that the selectivity of VP1 for host cell receptors and replication fidelity during replication are important individual factors in the induction of differences in pathogenicity in the host, as well as in the severity of outbreaks in the field.

**Table 13.** Clinical scores in cattle infected with FMDV O/JPN/2000 or O/JPN/2010

| Inoculated virus | Clinical sign | | Inoculated #1 | Contact #1 | Inoculated #2 | Contact #2 |
|---|---|---|---|---|---|---|
| O/JPN/2000 | Pyrexia | | – | – | – | 8 |
| | Excess salivation | | 1[a] | – | 4 | – |
| | Vesicular development | | | | | |
| | Tongue | | 1 | – | 4 | – |
| | Forelimb | Right | 3 | – | 7 | – |
| | | Left | 3 | – | 6 | – |
| | Hindlimb | Right | 2 | – | 6 | – |
| | | Left | 2 | – | 6 | – |
| O/JPN/2010[b] (previously reported (32)) | Pyrexia | | – | 5 | 3 | 4 |
| | Excess salivation | | 3 | 4 | 2 | 6 |
| | Vesicular development | | | | | |
| | Tongue | | 1 | 5 | 1 | 6 |
| | Forelimb | Right | 5 | 5 | 3 | 6 |
| | | Left | 6 | 5 | 4 | 6 |
| | Hindlimb | Right | 3 | 5 | 3 | 6 |
| | | Left | 2 | 5 | 4 | 6 |

[a] The post-inoculation or post-contact day on which clinical signs were initially observed.

[b] Results of clinical signs on infection with O/JPN/2010 in this table have been published elsewhere (32).

–, not detected

**Table 14.** Comparison of amino acid sequences of O/JPN/2000 and O/JPN/2010

| Genome region | Length of amino acids | No. of differences |
|---|---|---|
| $L^{pro}$ | 201 | 24 |
| VP4 | 85 | 0 |
| VP2 | 218 | 10 |
| VP3 | 220 | 11 |
| VP1 | 213 | 17 |
| 2A | 16 | 1 |
| 2B | 154 | 3 |
| 2C | 318 | 9 |
| 3A | 153 | 9 |
| 3B | 71 | 6 |
| $3C^{pro}$ | 213 | 5 |
| $3D^{pol}$ | 471 | 11 |
| (Total) | (2,333) | (106) |

**Table 15.** Oligonucleotide primers used for construction of chimeric recombinants

| Recombinant | type[a] | Sequence | Nucleotide position[b] |
|---|---|---|---|
| 5'-UTR/2000 vSVL-f02 | F in | GCAGGCGGCCGCTTGAAAGGGGGCGTTAGGGTCTC | NotI + 1-23 (+) |
| | R in | TCCGTTGCGGGTAGTGAGGATGC | 1993-2015 (–) |
| IRES/2000 vSVL-f02 | F in | AACCACAAGATGAACCTTCACC | 509-530 (+) |
| | R in | GTGTACAACAAAGCGATGAAACAGTC | 1110-1135 (–) |
| P1-1st/2000 vSVL-f02 | F in | CTTTCTTCGACTGGGTCTACCAC | 1294-1316 (+) |
| | R in | GGTGTACGCGTAATCAGCCGCCG | 3097-3119 (–) |
| P1-2nd/2000 vSVL-f02 | F in | ACCAACTTCCTTGATGTGGCTGA | 2733-2755 (+) |
| | R in | ACGTCAGAGAAGAAGAAGGGCCC | 3954-3976 (–) |
| P2/2000 vSVL-f02 | F in | CCAACCCTGGGCCCTTCTTCTTC | 3946-3968 (+) |
| | R in | GCGGATCATGATCACTATGTTTGCC | 5579-5603 (–) |
| P3/2000 vSVL-f02 | F in | TCAGTTTGGTACTGCCCACCTGA | 4827-4849 (+) |
| | R in | ATTTTCACTCCTACGGTGTC | 8139-8158 (–) |
| VP1/2000 vSVL-f02 | F in | TCGGCAACAGACCACCTCCACAGGTGAGTCGGCTGA | 3260-3295 (+) |
| | R in | CAGATCAAAGTTCAAAAGCTGTTTCACAGGCGCCA | 3886-3920 (–) |
| | F vec | TTGAACTTTGATCTGCTCAAGTTGGCA | 3906-3932 (+) |
| | R vec | GTGGTCTGTTGCCGAGCGTCCACAGGCA | 3247-3274 (–) |
| 3A-B/2000 vSVL-f02 | F in | CAATTCCTTCCCAAAAGGCTGTACTGTA | 5377-5404 (+) |
| | R in | GGGGGCACCACTCTCAGTGACAAT | 6033-6056 (–) |
| | F vec | GAGAGTGGTGCCCCCCCGACCGA | 6042-6064 (+) |
| | R vec | TTTGGGAAGGAATTGAGATCTGCTTGA | 5365-5391 (–) |

**Table 15.** (Continued)

| Recombinant | type[a] | Sequence | Nucleotide position[b] |
|---|---|---|---|
| 3C/2000 vSVL-f02 | F in | TTGATCGTCACCGAGAGTGGT | 6030-6050 (+) |
| | R in | CTCGTGGTGTGGTTCGGGGTCGATGTGT | 6656-6683 (–) |
| | F vec | GAACCACACCACGAGGGGTTGATCGTA | 6669-6695 (+) |
| | R vec | CTCGGTGACGATCAAGTTCCTAGCTTTCA | 6016-6044 (–) |
| 3D/2000 vSVL-f02 | F in | GAACCACACCACGAGGGATTGATAGTTGACACCA | 6669-6702 (+) |
| | R in | CTGAGAGATTATGCGTCACCGCACACGGCGTT | 8073-8104 (–) |
| | F vec | CGCATAATCTCTCAGATGTCACAATTGGCAGA | 8090-8121 (+) |
| | R vec | CTCGTGGTGTGGTTCAGGGTCGATGTGT | 6656-6683 (–) |

[a] F in, Forward primer for insert gene; R vec, Reverse primer for vector gene

[b] Nucleotide position corresponds to the nucleotide sequence of O/JPN/2010 290-1E (LC036265)

**Table 16.** Amino acid differences between O/JPN/2000 and O/JPN/2010 in VP1

| aa No.[a] | 28 | 47 | **58** | 85 | 96 | **137-142** | **153** | **158** | 185 | 194 | 198 | 212 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| O/JPN/2000 | Q | Q | **A** | N | T | **GESPVT** | **Q** | **T** | T | I | E | L |
| O/JPN/2010 | H | S | **S** | D | A | **AGGSLP** | **P** | **P** | A | V | A | S |

[a] Amino acid numbers were annotated with the VP1 of O/JPN/2010-290/1E (LC036265). Nine amino acid differences located on the G-H loop receptor binding domain are written in bold.

**Table 17.** Amino acid differences between O/JPN/2000 and O/JPN/2010 in 3D

| aa No.[a] | 34 | 63 | 68 | 98 | 144 | 148 | 254 | 262 | 330 | 425 | 469 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| O/JPN/2000 | F | N | E | A | E | K | N | N | S | T | D |
| O/JPN/2010 | Y | D | P | I | Q | E | S | R | T | I | G |

[a] Amino acid numbers are annotated with the 3D of O/JPN/2010-290/1E (LC036265).

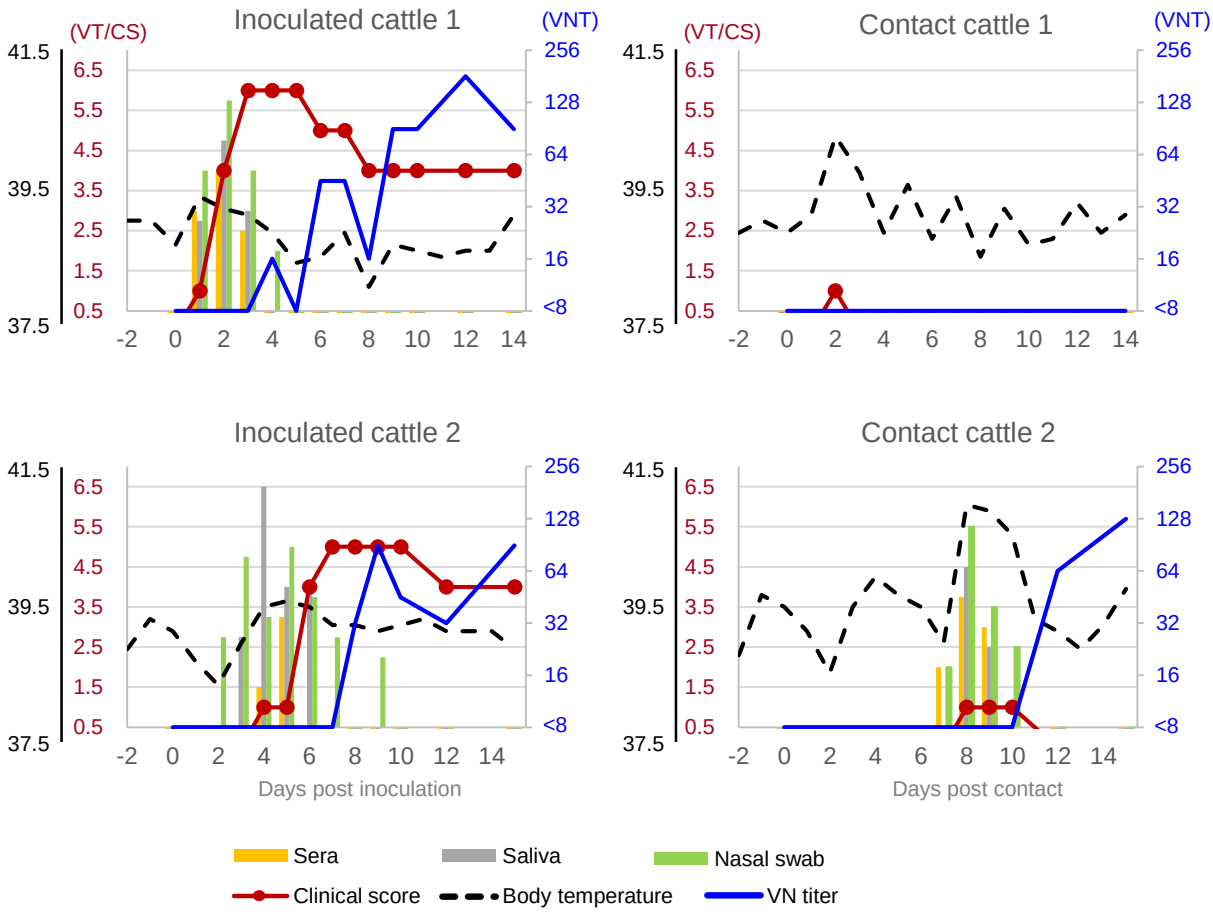


**Fig. 10. Time-course of infection in cattle inoculated with the FMDV O/JPN/2000 isolate or kept in contact with inoculated cattle.**

Times on the x-axes are given in dpi and dpc. Viral titers (VT, as $\log_{10}$ $TCID_{50}$/0.1ml) in sera and mouth and nasal swabs are shown together with the development of clinical signs (CS, score of 0 to 6) and rectal temperature (˚C) on the left y-axes; antibody titers measured in VNT are on the right y-axes.

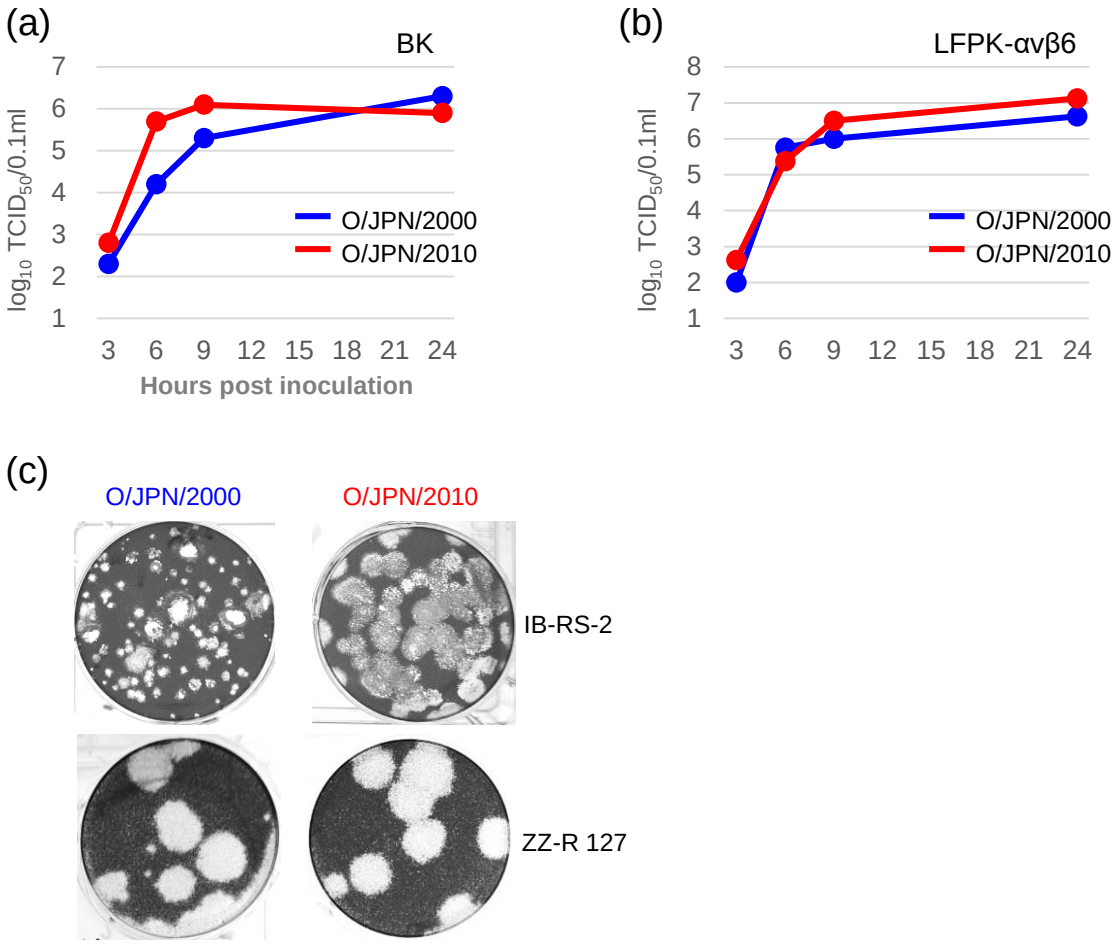


**Fig. 11. Growth characteristics of O/JPN/2000 and O/JPN/2010.**
One-step growth curves in (a) BK and (b) LFPK-αvβ6 cells. The cell monolayers were inoculated with each virus at a MOI of 0.1 and incubated at 37˚C. Samples of supernatant were collected at the indicated times and viral infectivity was determined. (c) Comparison of the plaque size of O/JPN/2000 and O/JPN/2010. The two strains were inoculated onto IB-RS-2 or ZZ-R 127 monolayer cells. The cultures were fixed 1 day after the inoculation and stained with crystal violet.

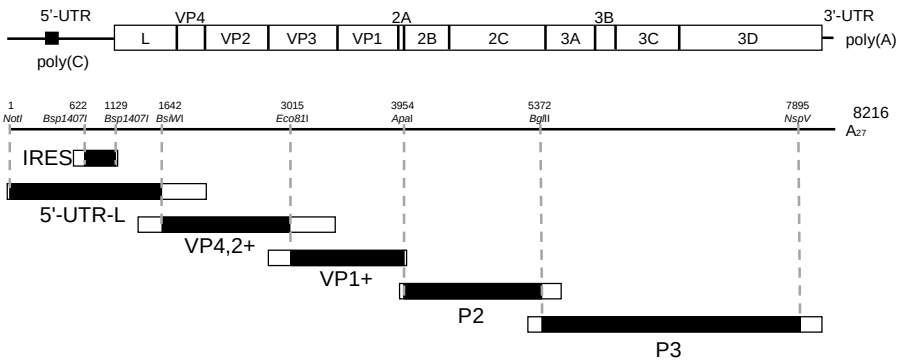


**Fig. 12. Insertion gene fragment of recombinant FMDV using O/JPN/2000 and O/JPN/2010.**

Each insertion gene of O/JPN/2000 amplified by PCR was ligated with the full-length infectious cDNA of O/JPN/2010 (pSVL-f02) by using appropriate restriction enzymes.

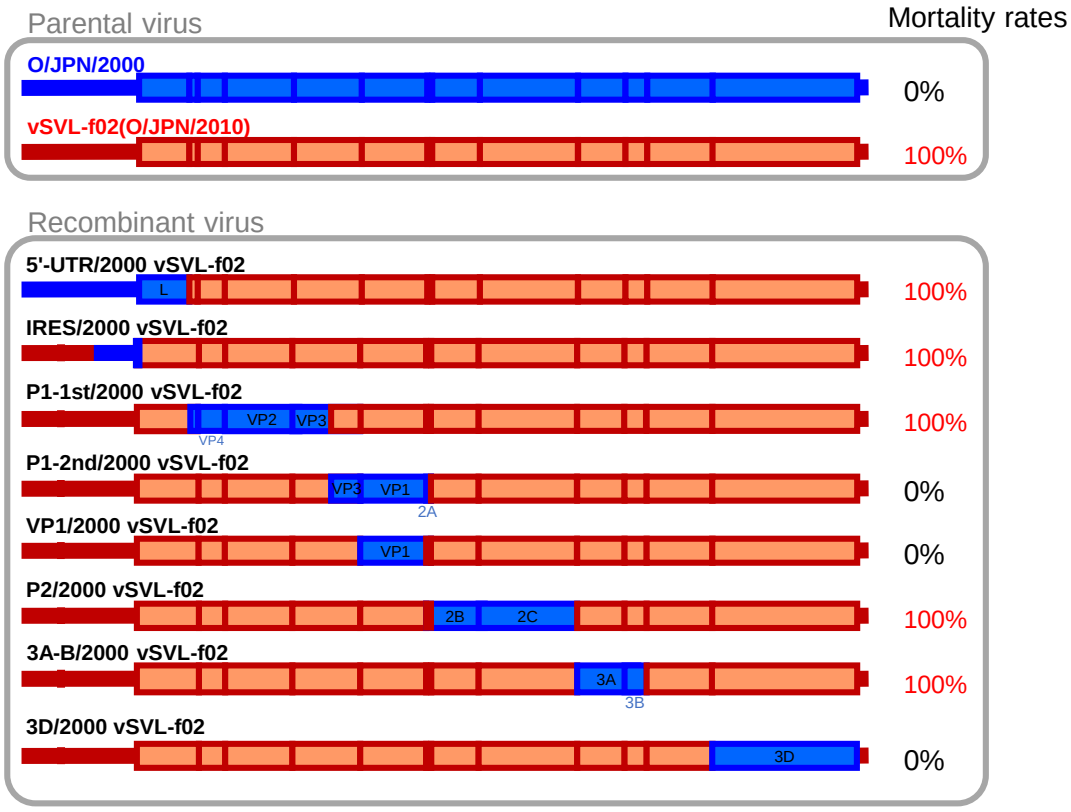


**Fig. 13. Schematic diagram and pathogenicity in suckling mice of recovered recombinant FMDVs between O/JPN/2000 and O/JPN/2010.**
Blue and red genes indicate genes from O/JPN/2000 and O/JPN/2010, respectively. Mortality rates of suckling mice inoculated with 10 $TCID_{50}$ of each recombinant virus are indicated at the right side.

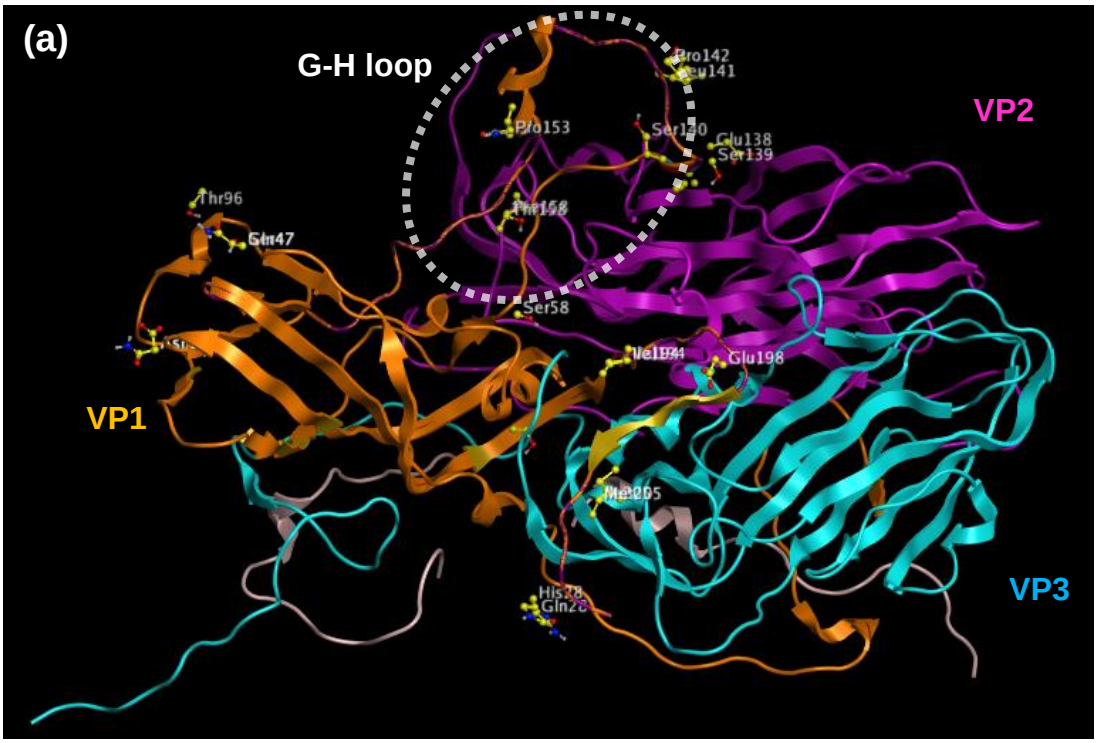


**Fig. 14. Positions of amino acid differences between O/JPN/2000 and O/JPN/2010 in three-dimensional structure.**
The amino acid differences between O/JPN/2000 and O/JPN/2010 were plotted as yellow dots on the three-dimensional structure of (a) VP1 or (b) 3D obtained from the Protein Data Bank (PDB; accession numbers 5NER and 4WZM, respectively) using MOE software.

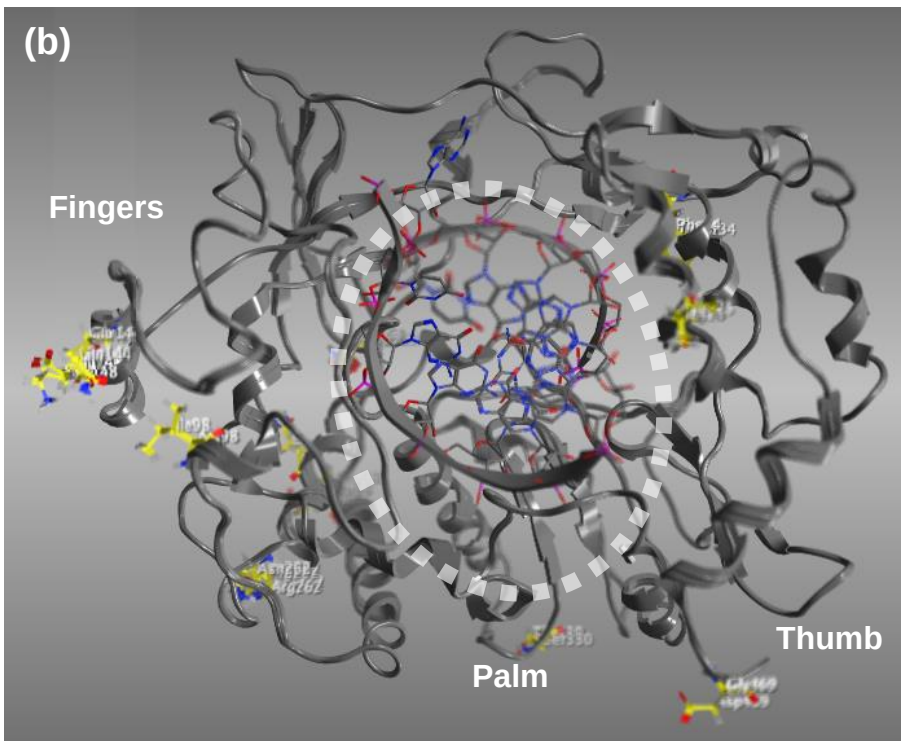


**Fig. 14. Continued**

# Conclusion

Individual FMDV strains reveal different degrees of infectivity and pathogenicity in host animals. The differences in severity among outbreaks might be ascribable to these differences in pathogenicity among FMDV strains. To date, however, few studies have described the genes responsible for its pathogenicity among multiple topotypes or the genome dynamics of FMDV over a widespread outbreak. In the present thesis, to investigate the molecular mechanisms underlying these differences, the author estimated the genome variability over an epidemic, infectivity in cell lines and natural hosts, and genetic determinants responsible for pathogenicity of FMDV isolated in Japan in 2010.

In Chapter I, the performance of RT-PCR assays using the primer set FM8/9 targeting the conserved 3D region of FMDV were compared with those of the RT-PCR assays described in the OIE manual. Compared with the RT-PCR assay using 1F/R primer set targeting 5'-UTR, the FM8/9 assay has much higher sensitivity for most FMDVs tested. In addition, this assay detected FMDV genes for longer periods from clinical samples of pigs and cows inoculated with FMDV. These results suggest that the FM8/9 RT-PCR assay is highly sensitive and is therefore suitable for the diagnosis of FMD. This assay was used for diagnosis during the FMD epidemic in Japan in 2010.

In Chapter II, to investigate how FMDV genetically evolves over a short period of an epidemic after initial introduction into an FMD-free area, whole L-fragment sequences of 104 FMDVs isolated from the 2010 epidemic in Japan, which continued for less than three months were determined and phylogenetically and comparatively analyzed. The results indicate that genetic substitutions of FMDV occurred gradually and constantly during the epidemic and characteristic mutations which related to atypical pathogenicity or host specificity could have been prevented by rapid eradication strategy.

In Chapter III, a full-length infectious cDNA clone of the genome of a FMDV isolated from the 2010 epidemic in Japan was constructed. Transfection of Cos-7 or IBRS-2 cells with this clone allowed the recovery of infectious virus. Virus recovered from transfected cells were confirmed to retain the *in vitro* characteristics and the *in vivo* pathogenicity of their parental strain.

In Chapter IV, genetic determinants of virulence between two FMDV isolates O/JPN/2000 and O/JPN/2010 which caused outbreaks of differing severity was elucidated. Viral growth of the two strains in cells was not remarkably different; however, O/JPN/2000 showed apparently low transmissibility in cattle and low virulence in suckling mice. To identify genes responsible for this difference in infectivity, genetic regions of the full-length cDNA of O/JPN/2010 were replaced with corresponding fragments of O/JPN/2000. Recombinants having either VP1 or 3D derived from O/JPN/2000 showed similar virulence in suckling mice with O/JPN/2000. It indicates that VP1, the outermost component of the virus particle, and 3D, an RNA-dependent RNA polymerase, are individually involved in the virulence of O/JPN/2010. Three-dimensional structural analysis and *in vitro* assay suggested that the selectivity of VP1 for host cell receptors and replication fidelity during replication were important individual factors in the induction of differences in pathogenicity in the host as well as in the severity of outbreaks in the field.

The findings described here provide new insights to understand the molecular mechanisms which underlie differences in pathogenicity beyond multiple topotypes of FMDV. These findings will aid the development of safe live vaccines and antivirals which obstruct viral infection in natural hosts.

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## Summary in Japanese（和文要旨）

口蹄疫はピコルナウイルス科アフトウイルス属に分類される口蹄疫ウイルスの感染による急性熱性伝染病である。ウイルスのゲノムは全長約 8,400 塩基のプラス極性一本鎖 RNA であり、5′末端より $L^{pro}$、VP1-4、2A、2B、2C、3A、3B、3C および 3D タンパクをコードしている。本ウイルスは互いにワクチンの効かない 7 つの血清型があるなど抗原性が多様である。本疾病は牛、水牛、豚、緬羊、山羊等、偶蹄類の家畜および野生動物に感染し、その伝染力は著しく強い。発病動物は鼻鏡を含む口周辺、蹄及び乳頭周辺部の皮膚や粘膜に水疱が形成され、摂食と歩行が困難となり、発育障害または泌乳障害により経済的価値を失う。水疱の中には大量のウイルスが含まれ、これが破れて周囲を汚染するほか、唾液、鼻汁、糞便、乳汁等からも排出され、エアロゾルによる空気伝播も起こる。一度発生すると、家畜及び畜産物の輸出入が厳しく制限されるため、社会経済的な被害は甚大となる。我が国では口蹄疫発生時には家畜伝染病予防法に基づき殺処分等の防疫措置がとられる。口蹄疫ウイルスの病原性や宿主域は株によって異なり、その性状の違いは畜産業にもたらす被害規模を大きく左右する。口蹄疫の病態についてはよく知られているが、ウイルスの流行時におけるゲノムの変異動態や病原性の分子基盤は未だ不明である。そこで本研究では、国内の畜産史上最も甚大な被害を及ぼした 2010 年の口蹄疫の発生について、原因ウイルスのゲノム変異動態

と病原性発現に関与する遺伝子を特定することにより、口蹄疫ウイルスの病原性の分子基盤の解明を試みた。

口蹄疫のような伝染力の強い疾病は、迅速かつ確実な診断に基づき、早期に防疫措置を講じることが重要である。RNA を増幅する Reverse transcription-PCR（RT-PCR）は、病原体を迅速に検出する方法の一つとして有用である。そこで、第 I 章では、口蹄疫ウイルスの株間で保存性の高いポリメラーゼ遺伝子を標的として設計されプライマー（FM8/9）について、その感度と特異度を国際獣疫事務局の推奨する 5′非翻訳領域を標的とするプライマー（1F/R）を用いた RT-PCR と比較した。全ての血清型を網羅する計 24 株の口蹄疫ウイルス RNA を各 RT-PCR に供して感度を検証した結果、FM8/9 を用いた RT-PCR は 1F/R を用いた場合と比較し、計 21 株で 4 から 6,300 倍高い感度を示した。また、口蹄疫ウイルスを実験的に感染させた豚、牛から経日的に採取した血清と唾液サンプルから RNA を抽出し、各 RT-PCR に供した結果、FM8/9 を用いた RT-PCR は顕著に高い検出率を示した。以上から、FM8/9 を用いた RT-PCR は病性鑑定において高感度にウイルス遺伝子を検出可能であることが明らかとなった。実際に、2010 年の国内発生においては、同等の感度であることが明らかとなったリアルタイム RT-PCR 法と併せて、迅速な診断法として重要な役割を果たした。

口蹄疫ウイルスはゲノム複製時における変異率が高く、その遺伝子は多様である。蔓延地域においては抗原変異株や豚でのみ症状を呈するなどの宿主特異性を持った変異株も確認されている。流行の時間枠中におけるウイルスの変異の動態を解明することは、適切な診断と防疫対策を講じる一助となる。第II章では、2010年宮崎県において口蹄疫の発生した 292 戸の材料から、104 株の塩基配列を決定し、比較解析した。海外のウイルスのゲノム全長配列と共に作成した分子系統樹において、104 株は一つのグループに分類されたことから、単一のウイルスが侵入、蔓延したことがわかった。104 株のゲノム全体の相同性は 99.56%～99.98%であり、完全に一致する配列はなかった。牛から分離されたウイルスと豚から分離されたウイルスに関してアミノ酸全長を比較解析したところ、宿主特異的置換は確認されなかった。各ウイルス株の塩基配列と採材日から算出した塩基置換率は $2.88 \times 10^{-5}$/塩基/日であり、既報の自然変異によるものと同等であった。迅速な防疫対応により発生規模を限局できたことで、性状の大きく異なるウイルスによる発生を防ぐことが出来たものと考えられる。各アミノ酸の変異割合の解析結果、ウイルスの外殻タンパク質を構成する VP1 と VP2 は変異が集中して遺伝的に多様であり、外殻タンパク質を架橋する VP4 および非構造タンパク質である 2C には変異が少なく遺伝的に安定していることが明らかとなった。

国内では2000年と2010年の二度にわたり口蹄疫が発生したが、2010年とは対照的に2000年の発生は4戸に抑えられた。現場での患畜の臨床症状から、原因ウイルスの宿主における病原性の差が、発生規模の差に関与したと考えられているがその詳細な分子基盤は明らかとなっていない。第III章では、2010年日本分離株のゲノムをもとに感染性cDNAを構築した。構築した感染性cDNAクローンは、2010年発生時の材料から得られたO/JPN/2010株（親ウイルス）のゲノムRNA全長のcDNAをプラスミドベクターに組み込んだものである。これを哺乳類動物細胞に導入することにより、感染性を有するウイルスを得ることが可能である。親ウイルスとの比較解析の結果、cDNA由来ウイルスは、親ウイルスと同様の細胞内増殖性および豚での病原性を示すことが確認された。これを用いて第IV章では、2000年および2010年に分離された2株を用いて遺伝子組換えウイルスを作出し、病原性に関与する遺伝子領域を探索した。2株間で遺伝子を網羅的に組換え、計8株のウイルスを回収した。親株O/JPN/2010およびO/JPN/2000を乳飲みマウスに接種した場合、致死率はそれぞれ100%、0%であった。これを基準として組換えウイルスの性状を解析したところ、O/JPN/2010のVP1および3Dをそれぞれに O/JPN/2000のものに組換えたウイルスを接種した群の致死率は0%だったが、その他の遺伝子領域を組換えたウイルスを接種した群の致死率は100%であった。ウイルス粒子の最外殻に位置して宿主内の主要レセプターとの結合および

免疫物質に作用する VP1、ならびに RNA の複製を担う 3D ポリメラーゼ遺伝子それぞれが、O/JPN/2010 の乳飲みマウスおよび自然宿主への病原性に大きく関わることが示唆された。VP1 の立体構造予測により、親株間でのアミノ酸の相違が主に細胞のレセプターとの結合部に位置することが分かった。一方で、2 つの親株についてウイルス複製時に起きる塩基置換率を解析したところ、O/JPN/2010 が O/JPN/2000 よりも 1.5 倍以上高かった。以上から、最外殻タンパクである VP1 のレセプター選択性、およびポリメラーゼによる RNA 複製の正確性が、口蹄疫ウイルスの宿主における病原性に重要な因子であることが分かった。

本研究は、口蹄疫ウイルスの遺伝子変異性と病原性発現機序の一端を分子レベルで解明したものである。これにより、口蹄疫ウイルスの病原性発現に寄与する遺伝子が明らかになり、安全で効果的なワクチンやそれらの遺伝子の機能を標的とする抗ウイルス剤の開発が可能となる。