Genetic Determinants of Altered Virulence of Type O Foot-and-Mouth Disease Virus

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ABSTRACT:

Under different circumstances, alteration of several viral genes could give an evolutionary advantage to the virus to maintain its prevalence in nature. In this study, a 70-nucleotide deletion in the Small-fragment (S-fragment) of viral 5’-untranslated region (5’-UTR) together with one amino acid insertion in the leader protein (L^{pro}) was identified that naturally occurred in several serotype O foot-and-mouth disease virus (FMDV) strains in China. The properties of two field serotype O FMDV strains, with or without the 70-nucleotide deletion in S-fragment and the amino acid insertion in L^{pro}, were compared in vitro and in vivo. Clinical manifestations of FMD were clearly observed in the cattle and pigs infected by the virus without the mutations. However, the virus with the mentioned mutations only caused FMD outcomes in pigs but not in cattle. To determine the role of the 70-nucleotide deletion in S-fragment and the single amino acid insertion in L^{pro} for the pathogenicity and host range of FMDV, four recombinant viruses, with complete genome, a 70-nucleotide deletion in the S-fragment or one single amino acid insertion in L^{pro}, or containing both the two mutations, were constructed and rescued. It showed that deletion of the 70-nucleotide in S-fragment or insertion of one amino acid (leucine) at the 10 site of L^{pro} partly decreased the viral pathogenicity of Mya-98 lineage virus in cattle and pigs. However, the virus with dual mutations only caused clinical disease in pigs but not in cattle. This suggested that the S-fragment and the L^{pro} are significantly associated with the virulence and host specificity of FMDV. The naturally occurred dual mutation in the
S-fragment and L^pro is a novel determinant of viral pathogenicity and host range for serotype O FMDV.

**IMPORTANCE**

FMD is probably the most important livestock disease in the world due to the severe economic consequences caused. Alteration of several viral genes could give virus selective advantage to maintain its prevalence in nature. Here, we identified that the 70 nucleotides deletion in the S-fragment combined with one single leucine insertion in the L^pro is a novel determinant of restricted growth on bovine cells, which significantly contributes to the altered virulence of serotype O FMDV to cattle. A synergistic and additive effect of the 70 nucleotides deletion in the S-fragment and the single leucine insertion in the L^pro on the virulence and host specificity of the virus was determined. These results will benefit the efforts to understand the viral pathogenicity mechanism and molecular characteristics of FMDV.
Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals. The rapid and extensive spread of FMD often results in trade restrictions and high economic losses (1). Foot-and-mouth disease virus (FMDV) is the etiological agent of FMD, which belongs to genus *Aphthovirus*, family *Picornaviridae*. FMDV includes seven major serotypes: O, A, C, Asia 1, SAT 1, SAT2 and SAT3, showing poor cross protective activity and highly genetic variability (2). Serotype O is the most common serotype worldwide, which also causes serious outbreaks in China. Most of FMDV strains infect all susceptible host species. However, some of FMDV strains have restricted host range. Such as, the serotype O Cathay strains only affect pigs (3). Several PanAsia topotype strains only caused clinical disease in cattle or only affected pigs (4). Nonstructural protein 3A has been determined as a genetic determinant of altered host tropism of FMDV outbreak in Taiwan in 1997 (3). A partial deletion in 3A was shown to attenuate serotype O FMDV in cattle (5).

The genome of FMDV is a single positive-sense strand of RNA of about 8.0 kb in length, which is artificially divided into 5′ untranslated region (UTR), the open reading frame (ORF) and 3′-UTR. The ORF sequence encodes four structural proteins (VP1, VP2, VP3 and VP4) and various non-structural proteins (L\(^{pro}\), 2A, 2B, 2C, 3A, 3B, 3C, 3D, 3AB or 3ABC) (1). FMDV leader protein (L\(^{pro}\)) is one of the main antagonistic factors of the virus which is widely known to cleave various host proteins and suppress host antiviral activity that contribute to virus replication (6). L\(^{pro}\) blocks host antiviral responses by means of different mechanisms, such as cleaving the host transcription factor and inhibit production of interferon (IFN)-α/β (6). L\(^{pro}\) has a
proteinase activity and self-cleaves from the nascent viral polyprotein precursor during FMDV replication and plays important roles in viral pathogenesis (7). Mutation of several functional domains of L<sup>pro</sup> dramatically impairs the pathogenicity of FMDV to the challenged hosts (8, 9).

5`UTR and 3`-UTR of FMDV are significantly involved in viral RNA replication and required for viral replication (10). FMDV 5`UTR contains several distinct elements with approximately 1,300 nucleotides (nt), including the Small-fragment (S-fragment), a poly(C) tract, several pseudoknots, a stable stem-loop structure termed the cis-acting replication element (cre) and the internal ribosome entry site (IRES) (11, 12). The first portion of the 5`UTR (about 350 nt) is the S-fragment consisting of a long stem-loop. The S-fragment of polio virus is only about 80 nucleotides (nt) long that is involved in viral RNA replication and stability (13, 14). As for the S-fragment of FMDV, previous studies suggested that it plays a role in viral replication and affects viral pathogenesis (15). poly(C) tract is about 150-200 nt in field FMDV strains that may regulate virus replication (16, 17). Our previous study indicates that the pseudoknots is related to viral pathogenicity and viral host range (18). The cre is about 55 nt in length that is required for FMDV RNA replication (12). The IRES is about 450 nt in length and directs the initiation of protein synthesis on the viral RNA (19, 20).

An unexpected deletion of 70 nucleotides within S-fragment of the 5`UTR of several Mya-98 lineage of the Southeast Asia topotype of serotype O FMDVs
isolated in China have been reported (21, 22). However, the role of this deletion in FMDV remains unknown (21). In the present study, we found that all the O/SEA/Mya-98 FMDV strains with the 70 nucleotides deletion were isolated from pigs. For all of the previously reported bovine-origin O/SEA/Mya-98 strains (with 5’UTR sequence information available in GenBank), no deletions were observed in the S-fragment. Meanwhile, we found that a single amino acid insertion existed in the Lpro of O/HKN/20/2010 which included the 70 nucleotides deletion within the S-fragment (22). This single amino acid insertion in the Lpro was in concurrence with the 70 nucleotides deletion in the S-fragment in all of these O/SEA/Mya-98 virus strains. To determine whether this deletion in the S-fragment and a single amino acid insertion in the Lpro has host specificity and affect virulence of the virus, the properties of two field O/SEA/Mya-98 lineage strains of O/BY/CHA/2010 (without the 70-nucleotide deletion and amino acid insertion) and O/Mya98/JX/2010 (containing the 70-nucleotide deletion and one amino acid insertion in the Lpro), were firstly investigated and compared. It indicated that O/BY/CHA/2010 affected both pigs and cattle; however, O/Mya98/JX/2010 only affected pigs and didn’t cause any clinical manifestations in cattle. The reverse genetics was subsequently used to produce genetically engineered chimeric viruses and define the genetic basis of the host specificity, and it was determined that the 70-nucleotide deletion in the S-fragment combined with the leucine insertion in the Lpro was a genetic determinant of virulence of O/SEA/Mya-98 FMDV that resulted in the bovine attenuation of the virus.
RESULTS

A 70-nucleotide deletion in the S-fragment within 5’UTR and a leucine/valine insertion in the L<sup>pro</sup> coexisted in several swine-origin O/SEA/Mya-98 FMDVs strains.

Our lab previously isolated an O/SEA/Mya-98 FMDV strain O/Mya98/JX/2010 (GenBank accession number: MN389541) from swine that included a 70-nucleotide deletion into the S-fragment in the 5’UTR of the viral genome. To further investigate the genomic characteristic of O/Mya98/JX/2010, the O/SEA/Mya-98 lineage FMDV complete genome sequences available in the GenBank were collected and analyzed. The comparison of the complete genome sequences revealed that another two viral strains HKN/20/2010 (GenBank accession number: HM229661) and O/GSLX/2010 (GenBank accession number: JQ900581) also included similar deletions within the S-fragment at the 148–217 (Fig. 1A). We also analyzed all the 5’UTR sequences of the O/SEA/Mya-98 lineage FMDV strains available in the GenBank, which showed that another 5 FMDV strains isolated in Hong Kong China in 2010 also included this deletion (Fig. 1A). This indicated that the 70-nucleotide deletion naturally occurred in the 5’UTR of several O/SEA/Mya-98 lineage FMDV strains. The polyprotein sequences of these strains (with complete genome sequences available in the GenBank) were further compared and analyzed. Interestingly, we found that the strains including the 70-nucleotide deletion also contained a 3-nucleotide insertion in the L gene. The amino acid sequences alignment of L<sup>pro</sup> indicated that the inserted
3-nucleotide encoded a leucine or valine at the position of 10 of L\textsuperscript{pro}, and O/Mya98/JX/2010 was similar as HKN/20/2010 containing a leucine insertion within L\textsuperscript{pro} (Fig. 1B).

O/Mya98/JX/2010 strain caused clinical signs in pigs but not in cattle

Ten amino acids deletion in 3A protein has been identified as an altered virulence and host tropism of serotype O Cathay topotype FMDV (3), and previous studies also suggest that there are also some other viral determinants that might be associated with the virulence and host range specificity of serotype O FMDV (23, 24). In the present study, the O/SEA/Mya-98 lineage FMDV strains with the 70-nucleotide deletion in the S-fragment of 5´UTR were all isolated from pigs, which implied that these strains possibly had a restricted host range that only affected pigs. To compare and evaluate the susceptible host species and virulence of the viruses with and without the observed mutations, two O/SEA/Mya-98 viruses, O/Mya98/JX/2010 (including the 70-nucleotide deletion within the 5´UTR and a leucine insertion in the L\textsuperscript{pro}) and O/BY/CHA/2010 (without the nucleotide deletion within the 5´UTR and amino acid insertion in the L\textsuperscript{pro}; GenBank accession number: JN998085), were used to carry out further studies.

10\textsuperscript{7}TCID\textsubscript{50}/animal of either FMDV was used to inoculate animals. It was a high dose of FMDV that could cause clear clinical outcome of FMD in both cattle and pigs.
Five cattle and 5 pigs were challenged with O/Mya98/JX/2010, and another 5 cattle and 5 pigs were challenged with O/BY/CHA/2010. All the challenged cattle and pigs were monitored daily for clinical signs of disease for two weeks (Fig. 2). The clinical scores were recorded to describe the clinical signs of disease as previously described (27). Briefly, one or more lesions per foot, is recorded as 1 point; the mouth, nostril, or tongue lesion beyond inoculation site, is recorded as 1 point. The maximum score per animal is 5. It was observed that the 5 cattle challenged with O/Mya98/JX/2010 showed no signs of clinical disease. In contrast, the 5 cattle challenged with O/BY/CHA/2010 revealed significant clinical signs of FMD (foot lesions) (Fig 2A). All the pigs challenged with O/Mya98/JX/2010 or O/BY/CHA/2010 developed typical clinical manifestations of FMD; however, the O/Mya98/JX/2010 induced slightly impaired or delayed clinical signs comparing to O/BY/CHA/2010. O/Mya98/JX/2010 caused clinical scores of 0-2 at the 4 days post-infection (dpi), and O/BY/CHA/2010 caused clinical scores of 3-4 at 4 dpi in the challenged pigs (Fig. 2B).

The viral RNAs in the blood and several organ tissues accessible to the virus (submandibular lymph nodes and tonsil tissues) were also detected to evaluate the infection status of the viruses in the challenged animals. Viral RNA was undetectable in the blood and organ tissues from the 5 cattle challenged with O/Mya98/JX/2010, and we could not recover O/Mya98/JX/2010 from these cattle. Whereas, the viral RNA was detectable in the blood and organ tissues from the 5 cattle challenged with O/BY/CHA/2010 (Fig. 2A), and the virus could also be recovered. As for the pigs, the
viral RNA was detectable from the viral replication organ tissues and blood of all pigs after viral challenge. However, the pigs challenged with O/Mya98/JX/2010 revealed a shorter viral RNA duration phase (Fig. 2B). Viral RNA existed in the blood of O/Mya98/JX/2010-challenged pigs from 1 to 7 dpi, and it could not be detected after 7-9 dpi. In the pigs challenged by O/BY/CHA/2010, the viral RNA disappeared after 9-11 dpi.

FMDV specific antibody titers and antibody against nonstructural protein (NSP) 3ABC were further detected. As shown in table 1, all the animals inoculated with O/BY/CHA/2010 developed significant levels of FMDV-specific antibodies starting at 5 to 9 dpi. However, the cattle challenged by O/Mya98/JX/2010 did not develop significant levels of FMDV-specific antibodies. All the animals revealed detectable NSP 3ABC antibodies except for the cattle challenged by O/Mya98/JX/2010. These results suggested that O/Mya98/JX/2010 only affected pigs but not cattle.

**Construction of the recombinant viruses with or without the mutations in the S-fragment and L<sup>pro</sup>**.

To investigate whether the 70-nucleotide deletion in S-fragment and the leucine insertion in L<sup>pro</sup> affect the host range of the serotype O FMDV, O/BY/CHA/2010 strain that presented the ability to affect both cattle and pigs was used for further study. Four recombinant viruses, rO-D70 (with the 70-nucleotide deletion in S-fragment), rO-L10 (with the leucine insertion in L<sup>pro</sup>), rO-D70-L10 (included the 70-nucleotide
deletion in S-fragment together with the leucine insertion in L<sup>pro</sup>) and rO strain (without the 70-nucleotide deletion and the leucine insertion), were rescued based on O/BY/CHA/2010 using the reverse genetic system. The characteristics of the 4 rescued viruses were shown in Fig. 3. We constructed a plasmid prO and three derivatives of plasmid prO (pr-D70, pr-L10 and pr-D70-L10) containing a 70-nt deletion in the S-fragment, a leucine insertion in the L<sup>pro</sup>, or a 70-nucleotide deletion combined with the leucine insertion in the L<sup>pro</sup>. Transfection of prO plasmid in BHK-21 cells generated a wildtype O/BY/CHA/2010 FMDV with a full-length S-fragment region and a wildtype L<sup>pro</sup> (rO), while transfection of pr-D70 generated a mutant O/BY/CHA/2010 strain with a 70-nucleotide deletion in the S-fragment region (rO-D70), transfection of pr-L10 generated a mutant O/BY/CHA/2010 strain with a leucine insertion at the position of 10 of L<sup>pro</sup> (rO-L10), and transfection of pr-D70-L10 generated a mutant O/BY/CHA/2010 strain with a 70-nucleotide deletion in the S-fragment region and a leucine insertion at the position of 10 of L<sup>pro</sup> (rO-D70-L10), respectively (Fig. 3A). An indirect immunofluorescence assay (IFA) was carried out using the polyclonal antibodies specific for FMDV to identify the rescued viruses in the BHK-21 cells, and clear green fluorescence was observed in the cells infected by the rescued viruses (data not shown). The plaque-forming assay revealed that all the 4 rescued viruses caused significant visible CPE in BHK-21 cells (Fig. 3B). These data suggested that the 4 viruses had been successfully rescued. The sequences of the S fragment and L gene of the 4 rescued viruses were determined and analyzed which confirmed the successful introduction of the designed deletion or
insertion in the viruses (Fig. 3C and 3D). To further confirm that, apart from the
70-nucleotide deletion in the S-fragment and a leucine insertion in the L\textsuperscript{pro}, the other
regions of the 4 viruses were completely identical, and the introduced mutations were
stable in the viral genome, the viral genome sequences of the 4th passage progeny
viruses in BHK-21 cells were determined and compared. The results showed that the
progeny viruses stably hold the designed mutations. Apart from the designed deletion
or/and insertion, some synonymous substitutions were found in the polyprotein
coding sequence. However, no amino acid mutation, deletion or insertion was
observed, which suggested that there was no difference among the viral proteins and
noncoding regions of the four viruses except for the designed deletion or/and
insertion.

The 70-nucleotide deletion in the S-fragment and a leucine insertion in the L\textsuperscript{pro}
decreased viral replication of O/BY/CHA/2010 in bovine-derived BTY cells.

The coexistence of the 70-nucleotide deletion in the S-fragment and one leucine
insertion in the L\textsuperscript{pro} was observed in O/Mya98/JX/2010 comparing to
O/BY/CHA/2010. To investigate the role of the 70-nucleotide deletion in S-fragment
and the single leucine insertion in L\textsuperscript{pro} on the pathogenicity of FMDV, the 4 rescued
viruses above were used to evaluate their replication ability in different cell lines in
vitro. Growth characteristics of the 4 viruses in BHK-21 cells, swine-derived PK-15
and IBRS-2 cells, and bovine-derived BTY cells were determined. One-step growth
curves showed that there was no remarkable difference in the viral titers among the four viruses in BHK-21 cells (Fig. 4A). In the PK-15 cells and IBRS-2 cells, the dual-mutation virus rO-D70-L10 revealed slower growth ability compared to the other three viruses (Fig. 4B and 4C). However, in the BTY cells, rO-D70-L10 showed significant replicative disadvantages over rO, rO-D70 and rO-L10, and caused clearly decreased cytopathogenic effect (Fig. 4D). These results indicated that the 70-nucleotide deletion in the S-fragment combined with the leucine insertion in the L$^{pro}$ contributed to the restricted growth of the virus on bovine cells.

The 70-nucleotide deletion in S-fragment together with the leucine insertion in L$^{pro}$ contributed to decreased pathogenicity of O/BY/CHA/2010 in cattle.

To investigate whether the 70-nucleotide deletion in the S-fragment and the leucine insertion in the L$^{pro}$ contributed to the bovine attenuation of rO in vivo, the 4 rescued viruses (rO, rO-D70, rO-L10 and rO-D70-L10) were further used for animal challenge experiments. Four groups of animals, with each group included 5 cattle and 5 pigs, were inoculated with $10^7$TCID$_{50}$/animal of different viruses. Meanwhile, an additional group of 5 cattle were challenged with a 10-fold higher dose of rO-D70-L10 ($10^8$TCID$_{50}$/cattle) to confirm its virulence in cattle. “RNAemia” is defined as the detection of FMDV RNA in blood samples of challenged animals. All the pigs challenged with rO, rO-D70 or rO-L10 manifested with RNAemia. rO-D70, rO-L10 and rO-D70-L10 showed decreased pathogenicity in pigs compared to rO, and
rO-D70-L10 showed the lowest pathogenicity. One of the pigs (no. 0004) challenged by rO-D70-L10 showed absence of RNAemia and clinical signs of disease (Fig. 5). However, in the cattle, rO-D70-L10 did not cause visible clinical signs of disease and RNAemia in all of the challenged animals (Fig. 6A), even at high dose of inoculum (10^8 TCID_{50}/cattle) (Fig. 6B). Besides, it revealed that rO-D70 and rO-L10 caused a significantly impaired clinical signs of disease and RNAemia in cattle compared to rO (Fig. 6A). This suggested an additive effect of the 70 nucleotides deletion in the S-fragment with the single leucine insertion in the L^pro to decrease the virulence of rO.

FMDV specific antibody and NSP 3ABC antibodies were further measured to confirm the infectious status of the animals challenged by various viruses. The results showed that rO-D70-L10 did not develop significant levels of FMDV-specific antibodies in cattle and no detectable NSP 3ABC antibodies were observed (Table 2). The 5 cattle inoculated with a high dose of rO-D70-L10 (10^8 TCID_{50}/cattle) also showed undetectable NSP 3ABC antibodies and extremely low levels of FMDV-specific antibodies (Table 3). Both NSP 3ABC and FMDV-specific antibodies were detected in all of the challenged pigs except for one pig (No. 0004) that was challenged by rO-D70-L10 showing absence of NSP 3ABC antibodies (Table 2). All these data confirmed that there was a synergistic and additive effect of the 70-nucleotide deletion within S-fragment and a single leucine insertion in L^pro on the virulence of serotype O FMDV to cattle. Deletion of the 70 nucleotides from the 5' end and insertion of one leucine in the L^pro in the O/SEA/Mya-98 FMDV has resulted in a critical bovine attenuation of the virus.
DISCUSSION

Compared to other picornaviruses, FMDV has a wide host range (28). In different countries or areas, different animals play different roles as the natural epidemiological or maintenance host under various conditions. For example, African buffalo are an important maintenance host in Africa (1). The prevalent virus strains also determine the epidemiological hosts and viral distribution status due to that different virus strains may have different epidemiological characteristics and host range, such as the Cathay topotype serotype O FMDV that only causes clinical disease in pigs (4). The evolution and mutations in the viral genome sequences can mediate adaptation of FMDV to different hosts, resulting in changes in host cell specificity and antigenicity (29). For instance, the nonstructural protein 3A is identified as the primary determinant for the restricted host range and a C-terminal truncation is proved to be responsible for the bovine-attenuated phenotype (3). Therefore, monitoring the sequence changes in the viral genome of FMDV is very essential for clarifying the pathogenic and virulence changes of the virus.

During 2010–2011, the serotype O Mya-98 lineage FMDV caused a series of high profile FMD outbreaks in China (21, 30). An unexpected 70-nucleotide deletion within the S-fragment of 5'UTR was identified in several of these virus strains which was located at the positions of 148 to 217 in the 5' UTR. This deletion resulted in a shorter predicted RNA stem-loop for the S-fragment. However, the effect of this
deletion on the virulence of FMDV remains unclear. To explore the influence of the 70-nucleotide deletion in the viral genome on the virulence or pathogenicity of FMDV, a thorough analysis of the viral genome sequences of different O/SEA/Mya-98 FMDV strains was firstly carried out. The sequence alignment revealed that an amino acid insertion in the L^{pro} at the position of 10 was in concurrence with the 70-nucleotide deletion in the S-fragment in these mutant viruses, and interestingly all of these strains were isolated from pigs. Subsequently, we evaluated and compared the replication and pathogenicity of two different field O/SEA/Mya-98 lineage FMDV strains of O/Mya98/JX/2010 (with the 70-nucleotide deletion and the leucine insertion) and O/BY/CHA/2010 (without the nucleotide deletion or amino acid insertion) both in vitro and in vivo. We found that O/Mya98/JX/2010 showed significantly restricted growth on bovine cells, and it only caused disease in pigs but no cattle; whereas, O/BY/CHA/2010 could replicate well both on bovine and swine cells, and it affected both pigs and cattle. Thus, we speculated that the 70-nucleotide deletion in S-fragment, the single leucine insertion in L^{pro} or the combined mutation of both S-fragment and L^{pro} may have resulted in the restricted growth of O/Mya98/JX/2010 on bovine cells and contributed to its failure of infection to cattle.

To investigate the role of 70-nucleotide deletion and a single leucine insertion in FMDV, four recombinant serotype O viruses were generated based on O/BY/CHA/2010 strain that had been proved to possess the ability to affect both cattle and pigs. The four viruses, with complete S-fragment and wildtype L^{pro} (rO), with 70-nucleotide deletion in the S-fragment region (rO-D70), with single leucine
insertion at the position of 10 in the L\textsuperscript{pro} (rO-L10), or with the 70-nucleotide deletion combined with the leucine insertion in the L\textsuperscript{pro} (rO-D70-L10), were rescued and further studied. The replication and pathogenic characteristics of these viruses were evaluated \textit{in vitro} and \textit{in vivo}. It was very interesting that, neither the deletion of 70-nucleotide in the S-fragment nor the single leucine insertion in the L\textsuperscript{pro} alone was the complete host-range determinant of O/BY/CHA/2010. Single mutation of the two factors just moderately decreased the virulence of O/BY/CHA/2010 to cattle. Only the coexistence of the mutations in S-fragment and L\textsuperscript{pro} could result in significantly restricted virus growth of O/BY/CHA/2010 on bovine cells and the failure of infection of the virus to cattle. There was a synergistic and additive effect of the 70-nucleotide deletion within the S-fragment and the single leucine insertion in the L\textsuperscript{pro} on the viral virulence of O/BY/CHA/2010. This indicated that the synergistic effect between the non-coding region and viral proteins was also a factor involved in the determination of host-specificity of FMDV. A recent study showed that replacement of the VP1 or 3D gene in the genome of FMDV O/JPN/2010 strain by the corresponding fragment derived from O/JPN/2000 could remarkably alter the virulence of O/JPN/2010 in cattle and suckling mice (31), suggesting that multiple genes are involved in the virulence of FMDV. Whether the VP1 and 3D genes have a synergistic effect can be further studied.

The S-fragment is engaged in viral replication regulation, contributing to pathogenesis (15), different kinds of deletions within S-fragment have been observed in different serotype FMDVs (21, 32). However, the effect of these deletions on viral
pathogenicity was not determined. The comparison of the predicted secondary structures of S-fragment of O/HKN/20/2010 that included a 70- nucleotide deletion within the S-fragment and O/HKN/15/2010 that contained a complete S-fragment showed that both of the two viruses have a single stem-loop, however, it was 35 pairs shorter in the apex of O/HKN/20/2010 than the one for O/HKN/15/2010 (21). Whether this change affects viral RNA stability remains unknown. In this study, this deletion partly decreased the virulence of O/BY/CHA/2010 both in pigs and cattle, which implied that S fragment is associated with viral pathogenicity, and the nucleotides between 148 and 217 of S fragment is involved in this function.

$L^{pro}$ has two isoforms (termed Lab and Lb) that result from the initiation at two different start codons that are separated by 84 nucleotides (33). $L^{pro}$ is well known as an antagonistic factor to suppress host antiviral responses and it is significantly associated with viral pathogenicity in host cells or animals (6). The precise loss of the Lb coding sequences of FMDV results in different growth characteristics of the virus in different host cells (34). A putative SAP domain [scaffold-attachment factor (SAF)A and SAFB, apoptotic chromatin-condensation inducer in the nucleus (ACINUS), and protein inhibitor of activated STAT (PIAS) domain] has been identified in FMDV $L^{pro}$ (35). The mutation of two residues at positions of 55 and 58 within the SAP domain of $L^{pro}$ has been determined to result in the viral phenotype change (35, 36). The SAP domain mutant virus reveals a stronger inductive ability to induce the expression of several IFN stimulated genes and chemokines compared with the wild-type FMDV; and this mutant virus can not cause disease to the challenged
pigs (9). Therefore, it indicated that \( L^{\text{pro}} \) is significantly responsible for the viral pathogenicity in the host. A leucine insertion in the \( L^{\text{pro}} \) of O/BY/CHA/2010 in this study also decreased the virulence of the virus both in swine and cattle, which confirmed that \( L^{\text{pro}} \) is associated with the viral pathogenicity. Whether the mentioned insertion of leucine at the position of 10 of \( L^{\text{pro}} \) changed the function of \( L^{\text{pro}} \) remains unclear, and the involved mechanisms should be further studied.

In the present study, the 70-nucleotide deletion in S-fragment or the single leucine insertion in \( L^{\text{pro}} \) could moderately decrease the virulence of the virus to the challenged animals. The clinical data in Fig. 6A indicated that the mutant virus with the \( L^{\text{pro}} \) insertion caused less disease in cattle than the mutant virus with the 70-nucleotide deletion, while the both viruses were as virulent in swine (Fig. 5). It suggested that \( L^{\text{pro}} \) might be a very critical factor that had resulted in the altered host tropism of the virus in cattle. However, the coexistence of mutations in the two regions resulted in significantly decreased growth ability of the virus in bovine cells and contributed to infection failure to cattle. It indicated that the two mutations synergistically caused the significant change of the viral phenotype and decreased the virulence of the virus to cattle. The concurrence of the two mutations in the O/SEA/Mya-98 lineage FMDV resulted in the altered host range of the virus that enabled the pigs became the main epidemiological host, and the pathogenicity of the virus tended to be milder. Serotype O is the most common serotype in China. The evolution of these FMDVs to just affect pigs possibly due to that China is a densely pig-populated country. The high amounts of pigs can provide better condition for the propagation and prevalence of these
viruses. Besides, the decreased viral pathogenicity may benefit virus maintenance in the host, because the high pathogenicity or quick replication of the virus may leave the host unable to further support viral maintenance or reproduction (37-39). Under these circumstances, altered S-fragment and L genes could have a selective advantage. The 70-nucleotide deletion in S-fragment and a leucine insertion in L<sup>pro</sup> of O/SEA/Mya-98 lineage FMDVs may be beneficial to the existence and prevalence of the virus in pigs. In summary, this study identified a novel genetic determinant of altered virulence of serotype O FMDVs, generating data that will benefit the efforts to understand the viral pathogenicity mechanism of FMDV.

### Materials and Methods

**Ethics statement.** All the animal experiments were approved and conducted according to the requirements of the Gansu Animal Experiments Inspectorate and the Gansu Ethical Review Committee (Licence no.SYXX(GAN) 2010-003).

**Cells and viruses.** Baby hamster kidney cell (BHK-21), porcine kidney cell line IBRS-2 and PK-15, and Bovine thyroid cell (BTY) were grown at 37°C and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (Gibco). All the cells were maintained at in a humidified incubator containing 5% CO<sub>2</sub> at 37 °C. FMDV O/Mya98/JX/2010 strain was isolated from an infected pig in the Ganzhou city, Jiangxi province of China in April 2010. The genomic sequence of the O/Mya98/JX/2010 was determined and deposited in the
NCBI GenBank database (accession no. MN389541). O/BY/CHA/2010 strain (accession no. JN998085) was isolated from an infected pig in the Baiyun District of Guangzhou city, Guangdong Province in March 2010.

**RNA extraction and cDNA synthesis.** Total RNA was extracted from FMDV-infected cells or tissue samples from virus-infected animals using RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer’s specifications and used as templates for cDNA synthesis. SuperScript Moloney murine leukemia virus reverse transcriptase (Life Technologies, Carlsbad, CA) was used for the reverse transcription reactions, and random hexamers were used as primers. The mixtures were incubated at 42°C for 1 h to synthesize the first-strand cDNA. The cDNA was then used as template for PCR reaction.

**Real-time qPCR (RT-qPCR).** One-step RT-PCR was performed as previously described by Shaw et al (40). Briefly, a total volume of 25 μl reaction mixture consisting of 20 μl of RT-PCR master mix reagents (containing the primers) and 5 μl of RNA was added to the appropriate number of wells in a 96-well optical reaction plate (Stratagene, La Jolla, CA). The reaction was performed in an Mx4000 Sequence Detection System (Stratagene) with an optimized thermal cycling condition. The Stratagene® MxPro™ QPCR software was used for the results analysis and a CT value was assigned to each reaction as described previously (41). Samples with a CT value of 35 or less were considered positive for FMDV detection (25).

**Construction of the rO, rO-D70, rO-L10 and rO-D70-L10 infectious clones.** The
construction strategy for the rO, rO-D70, rO-L10, and rO-D70-L10 infectious clones was shown in Fig. 3. A novel RNA polymerase I and II driven plasmid-based reverse-genetics system has been developed by our lab previously (25). The full-length infectious clone containing the FMDV O/BY/CHA/2010 sequence was generated based on this reverse-genetics system and named as prO. The other 3 mutant viruses were generated based on prO. A DNA fragment including the partial 5’UTR of FMDV O/BY/CHA/2010 with the 70-nucleotide deletion as well as two restriction endonuclease enzymes EcoRI and AfeI sites was synthesized, and the fragment was replaced into the prO as previously described (42) to generate pr-D70. pr-L10 was constructed with similar strategy with KpnI and AflIII enzymes, and 3 nucleotides “TTG” were introduced into L gene. pr-D70-L10 was constructed based on the p-rO-D70, and the KpnI and AflIII enzymes were used to introduce the single leucine insertion at the indicated position. All the constructed plasmids were sequenced.

Viral rescue and identification. The purified plasmids prO, pr-D70, pr -L10, and pr-D70-L10 were prepared using QIAGEN Plasmid Midi Kits (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The plasmids were transfected into monolayer BHK-21 cells in 10 cm culture plates using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 48 h post transfection, the supernatants were harvested. The supernatants were frozen and thawed for three times, and then were centrifuged at 5000×g for 10 min at 4°C. The supernatants were blind passaged into BHK-21 cells for 4 times. The rescued viruses
were collected and identified after 4 consecutive passages in BHK-21 cells. The immunofluorescence assay, plaque titration assay and 50% tissue culture infectious dose (TCID\textsubscript{50}) assay were performed to confirm the successful rescue of the viruses. The viral genomes of the obtained rescued viruses were finally sequenced to ensure no mutations were introduced.

\textbf{TCID}_{50} \textbf{assay.} BHK-21 cells were seeded in 96-well cell culture plates with $4 \times 10^4$ cells per well. Titrations were made using serial 10-fold dilutions. The confluent monolayer cells were infected with $10^{-1} - 10^{-8}$ dilutions of the rescued virus. Replicates of 8 wells (1st to 10th column) in a 96-well plate were used for each virus dilution (100 µl/well). The cell plate was incubated at in a humidified incubator containing 5% CO\textsubscript{2} at 37 °C for 1 h. The cells were then washed with PBS for 3 times to remove the unabsorbed viruses. The infected cells were then maintained with DMEM medium supplemented with 1% fetal bovine serum for 3 days. For each plate, the number of wells at each dilution with (+) or without (−) a cytopathogenic effect was recorded. The 50% endpoint titer of the virus was determined to calculate the 50% tissue culture infectious dose (TCID\textsubscript{50}).

\textbf{Experimental infection of various viruses in cattle and pigs.} Liquid-phase blocking ELISA (LPBE, \texttt{http://www.oie.int/eng/normes/mmanual/A_00024.htm}) was performed according to the standard method of the OIE to screen candidate pigs and cattle for animal challenge experiments. All the healthy animals tested negative for FMDV antibodies were used in this study. The animals were housed in the disease-secure isolation facilities at Lanzhou veterinary research institute. The cattle
of 6- to 7-month and the pigs of 6-week of age were housed separate rooms. The animals used for challenge by field viruses include: group 1 (cattle 2012, 2013, 2014, 2015 and 2016; pigs, 0994, 0996, 0997, 2147 and 2148) challenged by O/Mya98/JX/2010; group 2 (cattle 2009, 2010, 2011, 2017 and 2018; pigs, 2149, 2150, 3780, 3781 and 3782) challenged by O/BY/CHA/2010. The animals used for challenge by recombinant viruses include: group 3 (cattle, 0008, 0013, 0016, 0017 and 0018; pigs, 0016, 0017, 0018, 0019 and 0020) challenged by rO; group 4 (cattle 0005, 0007, 0014, 0021 and 0022; pigs, 0006, 0007, 0008, 0009 and 0010) challenged by rO-D70; group 5 (cattle 0006, 0009, 0011, 0012 and 0015; pigs, 0011, 0012, 0013, 0014 and 0015) and group 6 (cattle 0010, 0019, 0020, 0023 and 0024; pigs, 0001, 0002, 0003, 0004 and 0005), were challenged by rO-L10 and rO-D70-L10, respectively. The cattle from each group were inoculated intradermally at six sites in the tongue, with $10^7$TCID$_{50}$ or $10^8$TCID$_{50}$ of each virus. The pigs from each group were inoculated intramuscularly with $10^7$TCID$_{50}$ of each virus. All the animals were monitored daily and the clinical signs were recorded by scoring. The clinical scores for the challenged animals were determined as previously described by Rieder et al (27). The detailed criteria are: mouth, nostril, or tongue lesion beyond inoculation site $= 1$; one lesion per foot=$1$; and the maximum score for the infected-animal is 5. The animals were quickly removed to a separate room when they developed clinical disease for the duration of the experiment. The clotted and heparinized blood samples were collected at the 1, 3, 5, 7, 9, 11 and 15 days post-inoculation (DPI). The collected samples were subjected to viral RNA or antibodies detection.
Antibodies detection. The presence of antibodies against FMDV structural protein (SP) and nonstructural protein (NSP) 3ABC in the blood samples were determined by LBPE and NSP 3ABC detection kit. LBPE was performed as the manual of diagnostic tests and vaccines for terrestrial animals (http://www.oie.int/eng/normes/mmanual/A_00024.htm). Antibodies against the nonstructural protein (NSP) 3ABC were detected using the 3ABC indirect enzyme-linked immunosorbent assay; the NSP 3ABC detection kits were prepared by Lanzhou veterinary research institute and the detailed procedure was performed as previously described (43).

Statistical analysis. All results were presented as the means with standard error (SE). The significance was analyzed using GraphPad Prism (version 5.0) software. Statistical significance was defined as a P-value of less than 0.05.

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Table 1. NSP serology detected by 3ABC kit and specific antibody titers measured by LPBE assay.

| Virus             | Antibody | Species | Days post-infection | Species | No. | Days post-infection |
|-------------------|----------|---------|---------------------|---------|-----|--------------------|
|                   |          |         | 1  | 5  | 7  | 9  | 11 | 13 | 15 | 1  | 5  | 7  | 9  | 11 | 13 | 15 |
| O/Mya/9/08/2010   | 3ABC     | cattle  | -  | -  | -  | -  | -  | -  | -  | pg | -  | -  | -  | -  | -  | -  | -  |
|                   |          |         | 2012 | 1.11 | 1.22 | 1.45 | 1.45 | 1.45 | 1.45 | pg | 0.994 | -  | -  | -  | -  | -  | -  | -  |
|                   |          |         | 2013 | <1:8 | 1.11 | 1.11 | 1.45 | 1.45 | 1.45 | 1.45 | pg | 0.996 | <1:8 | 1.180 | 1.720 | 1.720 | 1.720 | 1.720 |
|                   |          |         | 2014 | <1:8 | <1:8 | 1.11 | 1.22 | 1.45 | 1.45 | 1.45 | pg | 0.997 | <1:8 | 1.16 | 1.45 | 1.720 | 1.720 | 1.720 | 1.720 |
|                   |          |         | 2015 | <1:8 | <1:8 | 1.11 | 1.22 | 1.45 | 1.45 | 1.45 | pg | 0.997 | <1:8 | 1.16 | 1.45 | 1.720 | 1.720 | 1.720 | 1.720 |
|                   |          |         | 2016 | <1:8 | <1:8 | 1.11 | 1.22 | 1.45 | 1.45 | 1.45 | pg | 0.997 | <1:8 | 1.16 | 1.45 | 1.720 | 1.720 | 1.720 | 1.720 |
| OBY/CHA/2010      | 3ABC     | cattle  | 2009 | -  | -  | -  | -  | -  | -  | pg | 2.149 | -  | -  | -  | -  | -  | -  | -  |
|                   |          |         | 2010 | -  | -  | -  | -  | -  | -  | pg | 2.150 | -  | -  | -  | -  | -  | -  | -  |
|                   |          |         | 2011 | -  | -  | -  | -  | -  | -  | pg | 2.150 | -  | -  | -  | -  | -  | -  | -  |
|                   |          |         | 2017 | -  | -  | -  | -  | -  | -  | pg | 2.150 | -  | -  | -  | -  | -  | -  | -  |
|                   |          |         | 2018 | -  | -  | -  | -  | -  | -  | pg | 2.150 | -  | -  | -  | -  | -  | -  | -  |

Specific antibody cattle

| Virus             | Antibody | Species | Days post-infection | Species | No. | Days post-infection |
|-------------------|----------|---------|---------------------|---------|-----|--------------------|
|                   |          |         | 2009 | <1:8 | >1:1024 | >1:1024 | >1:1024 | >1:1024 | >1:1024 | pg | 2.149 | <1:8 | 1.22 | 1.720 | 1.720 | 1.720 | 1.720 | 1.720 |
|                   |          |         | 2010 | <1:8 | >1:1024 | >1:1024 | >1:1024 | >1:1024 | >1:1024 | pg | 2.150 | <1:8 | 1.22 | 1.720 | 1.720 | 1.720 | 1.720 | 1.720 |
|                   |          |         | 2011 | 1.11 | 1.190 | 1.720 | 1.1024 | >1:1024 | >1:1024 | pg | 2.150 | 1.11 | 1.390 | 1.360 | 1.360 | 1.360 | 1.360 | 1.360 |
|                   |          |         | 2017 | <1:8 | 1.190 | 1.720 | 1.1024 | >1:1024 | >1:1024 | pg | 2.150 | 1.11 | 1.390 | 1.360 | 1.360 | 1.360 | 1.360 | 1.360 |
|                   |          |         | 2018 | 1.11 | 1.360 | >1:1024 | >1:1024 | >1:1024 | >1:1024 | pg | 2.150 | <1:8 | 1.380 | 1.360 | 1.360 | 1.360 | 1.360 | 1.360 | 1.360 |
Table 2. NSP serology detected by 3ABC kit and specific antibody titers measured by LPBE assay.

| Virus | Antibody | Species | No. | Days post-infection | Species | No. |
|-------|----------|---------|-----|---------------------|---------|-----|
|       |          |         | 1   | 5                   |         | 1   |
|       |          |         | 7   | 9                   |         | 7   |
|       |          |         | 9   | 11                  |         | 9   |
|       |          |         | 13  | 15                  |         | 13  |
| rO    | 3ABC     | cattle  | 0008 | -                   | pg      | 0016 |
|       |          |         | 0015 | -                   |         | -   |
|       |          |         | 0016 | -                   |         | -   |
|       |          |         | 0017 | -                   |         | -   |
|       |          |         | 0018 | -                   |         | -   |
|       |          |         | 0019 | -                   |         | -   |
| Specific antibody | cattle |         | 0008 | <1:8 1:180 | pg      | 0016 |
|       |          |         | 0015 | 1:11 1:90 |         | 0017 |
|       |          |         | 0016 | <1:8 1:180 |         | 0017 |
|       |          |         | 0017 | 1:11 1:90 |         | 0017 |
|       |          |         | 0018 | <1:8 1:180 |         | 0017 |
|       |          |         | 0019 | 1:11 1:90 |         | 0017 |
| Specific antibody | cattle |         | 0005 | -                   | pg      | 0016 |
|       |          |         | 0007 | -                   |         | 0017 |
|       |          |         | 0014 | -                   |         | 0017 |
|       |          |         | 0023 | -                   |         | 0017 |
|       |          |         | 0022 | -                   |         | 0017 |
| Specific antibody | cattle |         | 0005 | <1:8 1:180 | pg      | 0006 |
|       |          |         | 0007 | <1:8 1:11 |         | 0006 |
|       |          |         | 0014 | 1:11 1:90 |         | 0006 |
|       |          |         | 0023 | -                   |         | 0006 |
|       |          |         | 0022 | -                   |         | 0006 |
| Specific antibody | cattle |         | 0006 | -                   | pg      | 0011 |
|       |          |         | 0007 | -                   |         | 0012 |
|       |          |         | 0014 | -                   |         | 0012 |
|       |          |         | 0015 | -                   |         | 0012 |
| Specific antibody |         |         | 0006 | <1:8 1:180 | pg      | 0011 |
|       |          |         | 0007 | 1:11 1:180 |         | 0012 |
|       |          |         | 0014 | <1:8 1:11 |         | 0012 |
|       |          |         | 0015 | <1:8 1:11 |         | 0012 |

Note: < denotes no reaction, + denotes reaction.
| Specific antibody | cattle | pig |
|-------------------|--------|-----|
| 0010              | <1:8   | 1:45|
| 0019              | <1:8   | 1:11|
| 0020              | <1:8   | 1:22|
| 0023              | <1:8   | 1:11|
| 0024              | <1:8   | 1:11|
| 003               | >1:1024|
| 004               | >1:1024|
| 005               | >1:1024|

Note: The table represents antibody titers with 
- <1:8 indicates antibody titer is below 1:8 
- 1:45 indicates antibody titer of 1:45 
- >1:1024 indicates antibody titer is greater than 1:1024

Specific antibody values for cattle and pig are provided in the table above.
Table 3. NSP serology detected by 3ABC kit and specific antibody titers measured by LPBE assay in the cattle challenged with high dose of rO-D70-L10.

| Virus         | Antibody | Species | No.  | Days post-infection |
|---------------|----------|---------|------|--------------------|
| rO-D70-L10    | 3ABC     | cattle  | 0025 | -                  | 5      | 7      | 9      | 11     | 13     | 15     |
|               |          |         | 0026 | -                  | -      | -      | -      | -      | -      | -      |
|               |          |         | 0027 | -                  | -      | -      | -      | -      | -      | -      |
|               |          |         | 0028 | -                  | -      | -      | -      | -      | -      | -      |
|               |          |         | 0029 | -                  | -      | -      | -      | -      | -      | -      |
| Specific antibody | cattle  |         | 0025 | <1:8               | <1:8   | <1:8   | 1:11   | 1:22   | 1:22   | 1:22   |
|               |          |         | 0026 | <1:8               | <1:8   | <1:8   | 1:22   | 1:45   | 1:45   | 1:45   |
|               |          |         | 0027 | <1:8               | <1:8   | 1:11   | 1:11   | 1:11   | 1:11   | 1:11   |
|               |          |         | 0028 | <1:8               | <1:8   | 1:11   | 1:22   | 1:45   | 1:45   | 1:45   |
|               |          |         | 0029 | <1:8               | <1:8   | 1:11   | 1:11   | 1:22   | 1:22   | 1:22   |
FIGURE LEGENDS

FIG 1. A 70-nucleotide deletion in the S-fragment within the 5’UTR and an amino acid insertion in the L\(^{pro}\) occurred naturally in several O/SEA/Mya-98 FMDVs strains. (A) Alignment of the 5’UTR sequences of O/SEA/Mya-98 lineage FMDV strains. A 70-nucleotide deletion in the S-fragment within the 5’UTR was identified in O/Mya98/JX/2010, HKN/20/2010, O/GSLX/2010, HKN/18/2010, HKN/6/2010, HKN/4/2010, HKN/1/2010, and HKN/19/2010 after comparison with other O/SEA/Mya-98 FMDVs strains. (B) Alignment of the O/SEA/Mya-98 lineage FMDV polyprotein amino acid sequences available in the GenBank. A leucine/valine insertion at the position of 10 of L\(^{pro}\) was identified in O/Mya98/JX/2010, HKN/20/2010 and O/GSLX/2010 strains which coexisted with the 70-nucleotide deletion in the S-fragment.

FIG 2. O/Mya98/JX/2010 strain was not pathogenic in cattle. (A) Ten cattle were challenged by intradermal inoculation with \(10^7\) TCID\(_{50}\)/animal of O/Mya98/JX/2010 or O/BY/CHA/2010. The clinical signs were monitored daily and viral RNA levels in the blood were measured at 1, 3, 5, 7, 9, 11, 13 and 15 dpc. The viral RNAs in the bovine submandibular lymph nodes and tonsil tissues were measured using qPCR at 15 dpc. “RNAemia” is defined as the detection of FMDV RNA in blood samples of challenged animals. The X axes show the day post-challenge. The left Y axes represent the viral RNA amounts in the blood and tissues; and the right Y axes represent the clinical scores at different days post-challenge (the grey bar). (B) Ten pigs were inoculated intramuscularly with \(10^7\)TCID\(_{50}\) of O/Mya98/JX/2010 or O/BY/CHA/2010. The clinical signs and viral RNAs in
the blood and the organ tissues accessible to the virus were determined as described above to evaluate the infection status of the viruses in pigs.

**FIG 3. Construction and rescue of four recombinant viruses.** (A) Schematic representation showing the construct of prO, pr-D70, pr-L10 and pr-D70-L10. (B) Plaque assays of rO, rO-D70, rO-L10 and rO-D70-L10 on BHK-21 cells. (C) Alignment of the 5'UTR region sequences of the 4 recombinant viruses and O/Mya98/JX/2010, O/BY/CHA/2010, as well as another O/SEA/Mya-98 lineage FMDV strain O/GZ/CHA/2010. (D) Alignment of the polyprotein sequences of the 4 recombinant viruses and O/Mya98/JX/2010, O/BY/CHA/2010 and O/GZ/CHA/2010. Blue lines present the leucine insertion in the L\(^{pro}\).

**FIG 4.** rO-D70-L10 showed significantly decreased infective ability for bovine cells compared to rO. One-step growth curves of the four recombinant viruses in BHK-21 (A), IBRS-2 (B), PK-15 (C), as well as BTY (D) cells. The cells were infected with each virus at an MOI of 0.5 and maintained at 37°C. Samples of supernatants were harvested at the indicated times and the viral titers were measured.

**FIG 5.** rO-D70-L10 showed slightly reduced pathogenicity in pigs compared with rO. Four groups of pigs, with each group included 5, were inoculated intramuscularly with \(10^7\) TCID\(_{50}\) of rO, rO-D70, rO-L10 and rO-D70-L10, respectively. The clinical signs were monitored daily and viral RNAs in the blood were measured at 1, 3, 5, 7, 9, 11, 13 and 15 dpc. The viral RNAs in the submandibular lymph nodes and tonsil tissues were measured using qPCR at 15 dpc. “RNAemia” is defined as the detection of FMDV RNA in blood samples of challenged animals. The X axes show the day post-challenge. The left Y axes represent the
viral RNA amounts in the blood and tissues; and the right Y axes represent the clinical scores at different days post-challenge (the grey bar).

**FIG 6.** **rO-D70-L10 did not cause clinical manifestations in cattle.** (A) Twenty cattle were challenged by intradermal inoculation with $10^7$ TCID$_{50}$/animal of rO, rO-D70, rO-L10 or rO-D70-L10. The clinical signs were monitored daily and viral RNA levels were measured at 1, 3, 5, 7, 9, 11, 13 and 15 dpc. The viral RNAs in the submandibular lymph nodes and tonsil tissues were measured using qPCR at 15 dpc. (B) Five cattle were challenged by intradermal inoculation with $10^8$ TCID$_{50}$/animal of rO-D70-L10. The clinical signs and viral RNAs in the blood and submandibular lymph nodes and tonsil tissues were determined as described above. “RNAemia” is defined as the detection of FMDV RNA in blood samples of challenged animals. The X axes show the day post-challenge. The left Y axes represent the viral RNA amounts in the blood and tissues; and the right Y axes represent the clinical scores at different days post-challenge (the grey bar).
| Accession Number | Description |
|------------------|-------------|
| M389541.1        | O-Maoy8-JX-2010-Swine |
| JX920061.1       | HN-20-2010-Swine |
| JX985981.1       | GGLX-2010-Swine |
| KF112997         | HN-18-2010-Cattle |
| KF112998         | HN-1-2010-Swine |
| KF112999         | HN-1-2010-Swine |
| KF112998         | HN-19-2010-Swine |
| JN980055.1       | O-GZ-CHA-2010-Cattle |
| JX979508.1       | 0-OH-Md88-3-P-Cattle |
| KF112985         | JPN-1-2010-Cattle |
| KF112986         | SKR-5-2010-Cattle |
| KF112987         | SKR-5-2010-Swine |
| KF112988         | BRK-31-2011-Cattle |
| KF112989         | BRK-31-2011-Swine |
| KF112990         | BRK-31-2011-Swine |
| KF112991         | BRK-31-2011-Swine |
| KF112992         | BRK-31-2011-Swine |
| KF112993         | BRK-31-2011-Swine |
| KF112994         | BRK-31-2011-Swine |
| KF112995         | BRK-31-2011-Swine |
| KF112996         | BRK-31-2011-Swine |
| KF112997         | BRK-31-2011-Swine |
| KF112998         | BRK-31-2011-Swine |
| KF112999         | BRK-31-2011-Swine |
| KF112998         | BRK-31-2011-Swine |
| KF112999         | BRK-31-2011-Swine |
| KF112998         | BRK-31-2011-Swine |
| KF112999         | BRK-31-2011-Swine |

**B. Majority**

| Accession Number | Description |
|------------------|-------------|
| M389541.1        | O-Maoy8-JX-2010-Swine |
| JMX23661.1       | HN-20-2010-Swine |
| JQ900581.1       | O-GLX-2010-Swine |
| JX985981.1       | O-BY-CHA-2010-Swine |
| JN980055.1       | O-GZ-CHA-2010-Cattle |
| KF112985         | JPN-1-2010-Cattle |
| KF112987         | SKR-5-2010-Swine |
| KF112988         | BRK-31-2011-Cattle |
| KF112989         | BRK-31-2011-Swine |
| KF112990         | BRK-31-2011-Swine |
| KF112991         | BRK-31-2011-Swine |
| KF112992         | BRK-31-2011-Swine |
| KF112993         | BRK-31-2011-Swine |
| KF112994         | BRK-31-2011-Swine |
| KF112995         | BRK-31-2011-Swine |
| KF112996         | BRK-31-2011-Swine |
| KF112997         | BRK-31-2011-Swine |
| KF112998         | BRK-31-2011-Swine |
| KF112999         | BRK-31-2011-Swine |
| KF112998         | BRK-31-2011-Swine |
| KF112999         | BRK-31-2011-Swine |
| KF112998         | BRK-31-2011-Swine |
| KF112999         | BRK-31-2011-Swine |

**C. Summary**

- Majority
  - Accession Number
  - Description
- B. Majority
  - Accession Number
  - Description
