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GENETIC DIVERSITY AND EVOLUTION

Polymerase Fidelity Contributes to Foot-and-Mouth Disease Virus Pathogenicity and Transmissibility *In Vivo*

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ABSTRACT The low fidelity of foot-and-mouth disease virus (FMDV) RNA-dependent RNA polymerase allows FMDV to exhibit high genetic diversity. Previously, we showed that the genetic diversity of FMDV plays an important role in virulence in suckling mice. Here, we mutated the amino acid residue Phe257, located in the finger domain of FMDV polymerase and conserved across FMDV serotypes, to a cysteine (F257C) to study the relationship between viral genetic diversity, virulence, and transmissibility in natural hosts. The single amino acid substitution in FMDV polymerase resulted in a high-fidelity virus variant, rF257C, with growth kinetics indistinguishable from those of wild-type (WT) virus in cell culture, but it displayed smaller plaques and impaired fitness in direct competition assays. Furthermore, we found that rF257C was attenuated *in vivo* in both suckling mice and pigs (one of its natural hosts). Importantly, contact exposure experiments showed that the rF257C virus exhibited reduced transmissibility compared to that of wild-type FMDV in the porcine model. This study provides evidence that FMDV genetic diversity is important for viral virulence and transmissibility in susceptible animals. Given that type O FMDV exhibits the highest genetic diversity among all seven serotypes of FMDV, we propose that the lower polymerase fidelity of the type O FMDV could contribute to its dominance worldwide.

IMPORTANCE Among the seven serotypes of FMDV, serotype O FMDV have the broadest distribution worldwide, which could be due to their high virulence and transmissibility induced by high genetic diversity. In this paper, we generated a single amino acid substitution FMDV variant with a high-fidelity polymerase associated with viral fitness, virulence, and transmissibility in a natural host. The results highlight that maintenance of viral population diversity is essential for interhost viral spread. This study provides evidence that higher genetic diversity of type O FMDV could increase both virulence and transmissibility, thus leading to their dominance in the global epidemic.

KEYWORDS foot-and-mouth disease virus, RdRp, attenuation, polymerase fidelity, transmissibility

Due to the low fidelity of the viral RNA-dependent RNA polymerase (RdRp), RNA viruses exhibit genetically diverse populations known as quasispecies (1–3). Accumulating evidence has shown that RNA virus variants with high-fidelity RdRps are attenuated *in vivo* (4–7), which are the results of the restricted quasispecies diversity. Alternatively, increased mutation frequency places higher than the usual number of genomic RNAs beyond a hypothetical error threshold. Thus, RNA viruses with too many mutations exhibiting low-fidelity RdRps were also attenuated (8–11). Additionally, changes in polymerase fidelity can impact the virulence of DNA viruses. For example,

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increased virulence of Marek's disease virus can be achieved by the formation of a genetically very diverse population at transmission sites (12). Therefore, maintaining genome diversity is essential for viruses to evolve rapidly and overcome bottlenecks during host-to-host transmission (13–15).

Foot-and-mouth disease (FMD) is an extremely contagious disease that affects domestic and wild cloven-hoofed animals, resulting in significant economic loss to livestock producers and farmers. Foot-and-mouth disease virus (FMDV), the causative agent of FMD, belongs to the Redrops *Aphthovirus* genus within the *Picornaviridae* family (16, 17). The virus exists as seven different serotypes, as follows: A, O, C, Asia1, South African Territories 1 (SAT1), SAT2, and SAT3 (16, 18). Among these seven serotypes, serotype O FMDV has the broadest distribution around the world (18) and it is divided into 11 genotypes based on phylogenetic analysis (19). In contrast, the type Asia1 serotype contains a single genotype, displaying the least genetic diversity among all FMDV serotypes (20, 21). Previously, we reported that the genetic diversity of type O FMDV is approximately 2.06-fold higher than that of type Asia1 FMDV (22–24). Recently, a murine norovirus high-fidelity polymerase I391L mutant was rescued based on other fidelity variants isolated in related picornaviruses. The I391L mutant showed impaired mouse-to-mouse transmission compared to wild-type (WT) virus, supporting the concept of genetic diversity in controlling infectivity and transmission of norovirus *in vivo* (7). Additionally, Chikungunya virus populations with greater genetic diversity are capable of rapid intrahost diversification during infection (25), suggesting that the high genetic diversity of a virus can increase its virulence. These results inspired us to investigate whether the lower polymerase fidelity and the higher genetic diversity presented by type O FMDV could explain its dominance among the seven serotypes of FMDV in globally circulating strains.

Studies have shown that polymerase mutations affecting nucleoside triphosphate (NTP) binding can alter RdRp fidelity (26–28), as do mutations near the NTP entry channel (6, 29–31). The conserved F257 residue within the hydrophobic cavity of the FMDV RdRp contributes to catalysis upon NTP binding (32). Thus, we hypothesized that mutating F257 would alter FMDV polymerase fidelity, resulting in viral attenuation and impaired transmissibility. Animal models have been widely used to examine the virulence of polymerase fidelity variants, and we previously demonstrated that FMDV mutants with either increased or decreased RdRp fidelity are attenuated in suckling mice (22–24). We successfully generated a recombinant serotype O FMDV mutant (rF257C) with growth kinetics in culture indistinguishable from the parental virus. Subsequently, we found that the F257C mutation resulted in a higher-fidelity FMDV variant that displayed less fitness in competition assays. We also show that the rF257C mutant is attenuated *in vivo* in both a suckling mouse model and in one of its natural hosts, the pig. Additionally, rF257C transmitted less efficiently between pigs compared to the wild-type strain, providing evidence that polymerase fidelity is a determinant of FMDV virulence and transmissibility in a natural host. Our study highlights the value of using natural hosts as ideal candidates for the evaluation of attenuated viruses and emphasizes the importance of FMDV genetic diversity in the rational design of FMD control strategies.

RESULTS

Generation of the FMDV RdRp mutant rF257C. Among our previously constructed FMDV 3D^{pol} mutants (8), none of them exhibited a high-fidelity polymerase phenotype. In addition, in the process of isolating type Asia1 and type O FMDV 3D^{pol} mutants by nucleoside analog selection (23, 24), we found that mutating the Met296 residue within a highly hydrophobic cavity, important for the incoming NTP, altered polymerase fidelity. Both Met296 and Phe257 are critical amino acid residues that form a highly hydrophobic cavity of FMDV RdRp (32). Sequence alignment revealed that the F257 in 3D^{pol} is highly conserved among all serotypes of FMDV strains available in GenBank (Fig. 1B). Thus, we hypothesized that residue F257 in the cavity could also be important for polymerase fidelity. The F257 residue, located in the finger domain of

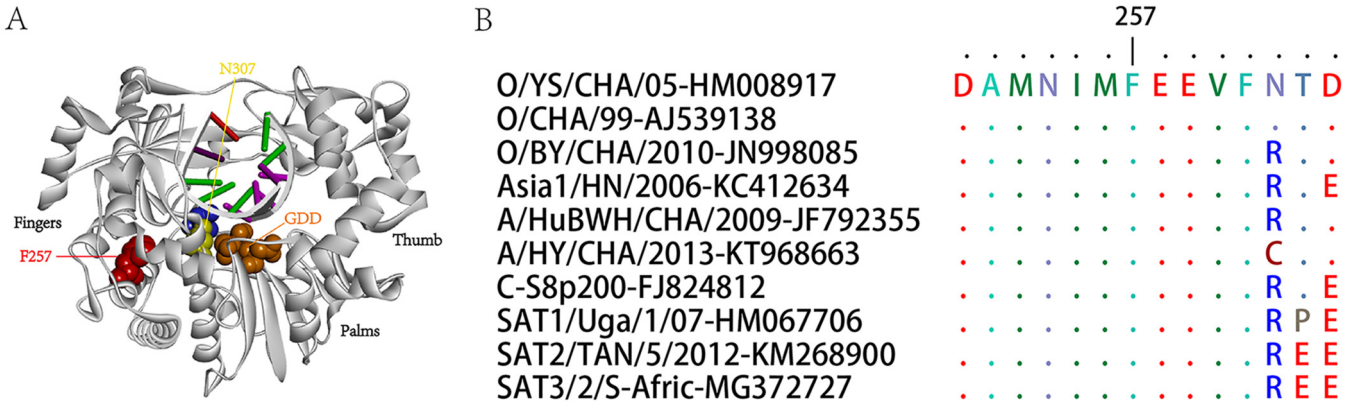

FIG 1 Structural overview of the FMDV polymerase. (A) The residue at position 257 in FMDV 3D^{pol}-RNA complex structure. The potential critical amino acid residues Asp245 and Asn307 in FMDV 3D^{pol} are colored blue and yellow, respectively. The active site (GDD motif, nucleotide-binding site) of 3D^{pol} is colored brown. (B) The alignment of the FMDV 3D^{pol} amino acid sequence available in GenBank revealed that the Phe257 is highly conserved in all serotypes of FMDV strains.

FMDV 3D^{pol} (Fig. 1A), was selected for mutagenesis using a serotype O FMDV infectious clone to generate a recombinant FMDV mutant based on the structure of FMDV polymerase (32–34). The F257 residue was replaced with the slightly smaller residue cysteine, and the recombinant FMDV 3D^{pol} mutant, designated rF257C, was successfully recovered after transfection on baby hamster kidney cell (BHK-21 clone 13) cultures. Both the rF257C and the wild-type (WT) virus showed a titer of $10^{6.75}$ 50% tissue culture infective dose ($TCID_{50}$)/ml.

The RdRp F257C mutation increases polymerase fidelity. The mutation frequencies of WT and the rF257C FMDV during infection were determined to assess their polymerase fidelity. For each viral population at passage 10, a 500-bp fragment of the capsid protein-encoding region from 70 to 80 individual clones was sequenced to determine the average number of mutations per 10^4 nucleotides (nt). The mutation frequencies of the 500-bp fragment from FMDV WT and rF257C were 8.77 and 6.25 mutation/10^4 nucleotides, respectively ($P < 0.05$ by a two-tailed Mann-Whitney U test) (Fig. 2A). These results demonstrated that rF257C produced significantly fewer mutations compared to FMDV WT, with a 1.4-fold increase in fidelity. To exclude the possibility that the reduced mutation frequencies in rF257C were the result of impaired viral replication, we compared the replication kinetics of the two viruses. Growth kinetics of rF257C under one-step growth conditions in BHK-21 cells and swine kidney epithelial cells (IBRS-2) cells revealed no significant differences relative to those of the

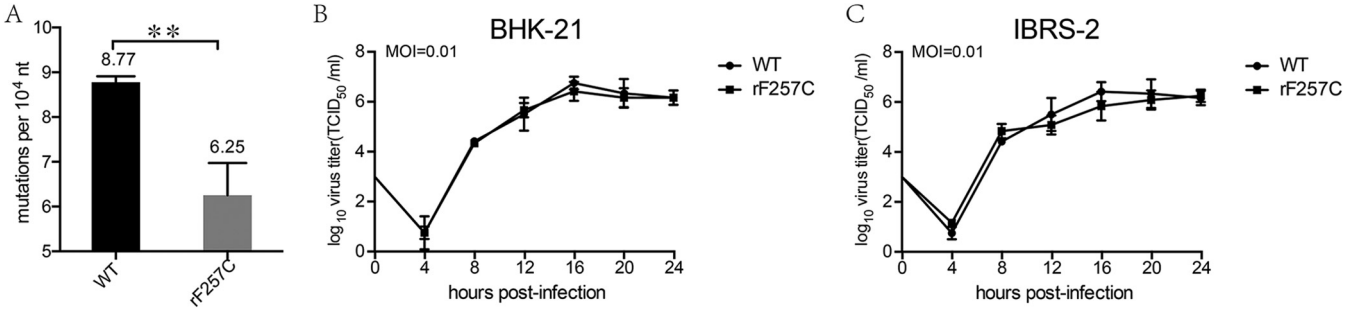

FIG 2 The FMDV rF257C mutant is a high-fidelity variant. (A) A mean of 70 partial P1 sequences (approximately 35,000 nucleotides per replicate) was obtained. The mean mutation frequencies (number of nucleotide mutations per 10,000 nucleotides sequenced) ± standard deviation (SD) represent the averages of all replicates. The same pattern of reduced mutation frequency for the polymerase mutant compared to the WT was observed for each replicate (two-tailed Mann-Whitney test, $n = 3$; *, $P < 0.05$; **, $P < 0.01$). (B and C) Replication kinetics of the FMDV RdRp high-fidelity mutant. BHK-21 and IBRS-2 cells were infected with the mutant rF257C or with FMDV WT at a multiplicity of infection (MOI) of 0.01. Titers of the virus harvested at different times were determined and expressed as 50% tissue culture infective dose ($TCID_{50}$). Mean values ± SD are shown (repeated-measures analysis of variance [ANOVA], $n = 3$; not significant [ns] for all mutants compared with WT).

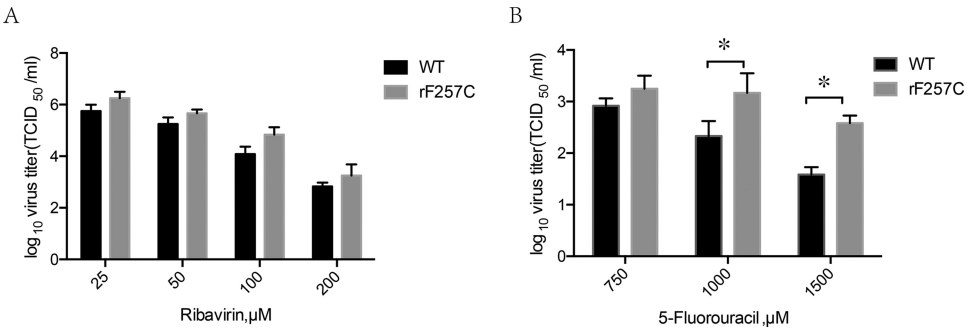

FIG 3 Virus mutagen sensitivity assay of FMDV strains. BHK-21 cells, treated with different concentrations of ribavirin (A) or 5-fluorouracil (B) or mock treated, were infected at an MOI of 0.01 for 72 h. Viral infectivity was determined by $TCID_{50}$ assay. Mean virus titers ± SD are shown (Student's *t* test, $n = 4$; *, $P < 0.05$; **, $P < 0.01$).

WT (Fig. 2B and C). Thus, the higher fidelity observed in the rF257C mutant is not due to replication defects.

We evaluated the resistance of rF257C to the nucleoside analogs ribavirin and 5-fluorouracil (5-FU), compared to that of the WT virus (Fig. 3). Our results showed that there was a trend of rF257C being more resistant than the WT to ribavirin, although no significant difference was observed when rF257C or the parental virus was exposed to various concentrations of the nucleoside analog ribavirin (Fig. 3A). In contrast, rF257C showed resistance to higher 5-FU concentrations of 1,000 μM and 1,500 μM (Fig. 3B). These results suggested that increased polymerase fidelity is associated with increased 5-FU mutagen resistance.

The rF257C virus exhibits reduced fitness and smaller plaque size. High-fidelity RdRp variants with decreased error rates during virus replication result in decreased quasispecies. Thus, most high-fidelity RdRp variants present reduced fitness *in vitro* (35–38). To assess whether the rF257C causes a fitness deficit, we performed a direct competition assay. The specific codon at the corresponding RdRp position was used to differentiate the mutant from the WT. After three passages in BHK-21 cells, the chromatogram peak corresponding to the rF257C mutation was extremely reduced relative to WT (Fig. 4A). This result shows that rF257C exhibits an apparent fitness disadvantage compared to the WT, even though the replication kinetics of the virus was similar to that of the FMDV WT in these cells (Fig. 2B).

The reduced fitness displayed by rF257C in competition assays suggested that rF257C could exhibit a smaller plaque. Since viral plaque size is often an indicator of virus replication rate and attenuation, we investigated whether there are plaque size differences between the WT and rF257C in BHK-21 cells. Results showed that the average diameters of the WT and the rF257C mutant are 2.3 ± 0.16 mm and 1.4 ± 0.11 mm, respectively, indicating that the rF257C mutant produces smaller

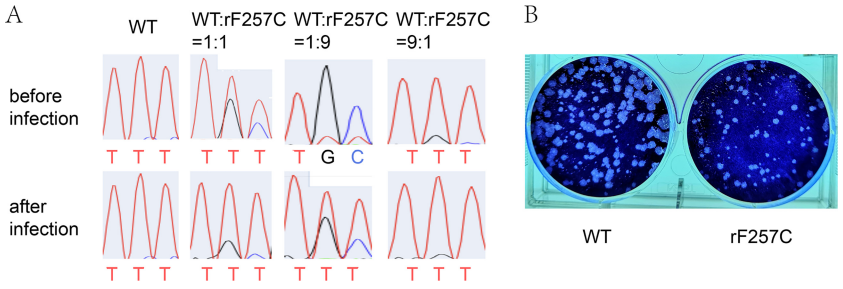

FIG 4 Fitness and plaque morphology of the rF257C mutant. (A) The rF257C mutant was mixed with the WT at a ratio of 9:1, 1:1, or 1:9 to inoculate BHK-21 and IBRS-2 cells at an MOI of 0.1 for three passages, after which the $3D^{pol}$ region was sequenced. The abundance of each competitor was measured as the height of the nucleotide peak for the mutant (G or C nucleotide) or the WT (T nucleotide) in sequencing chromatograms. (B) The plaque phenotypes of WT and rF257C were monitored in BHK-21 cells.

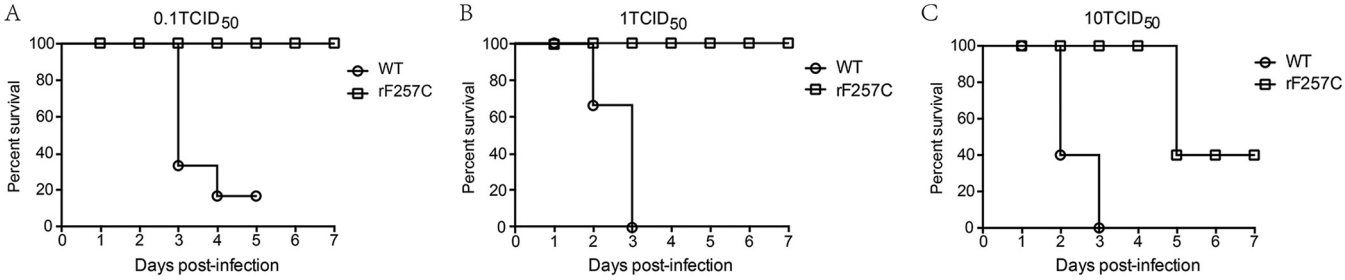

FIG 5 The rF257C is attenuated in BALB/c suckling mice. A total of 3 groups of 3-day-old BALB/c suckling mice were inoculated cervicodorsally with 200 μl of each mutant or WT FMDV diluted to 0.1 $TCID_{50}$ (A), 1 $TCID_{50}$ (B), or 10 $TCID_{50}$ (C). Animal deaths were scored for up to 7 days after inoculation, and survivors were euthanized ($n = 6$ to 9 mice for each group).

plaques than those produced by the WT virus (Fig. 4B). It has been demonstrated that engineered FMDV high- and low-fidelity variants exhibit similar growth kinetics but somewhat smaller plaque sizes than those of the WT strain in BHK-21 cells (31). More importantly, cell culture adaptation of FMDV requires binding to cell surface heparan sulfate, which also affects plaque size (39–41), indicating that cell adaption plays an important role in FMDV plaque size. In this study, both the WT and rF257C were cell-adapted viruses, so the FMDV WT with high genetic diversity produced more cell-adapted viruses than the high-fidelity virus presents fewer mutations during serial passage. Therefore, cell adaption could be one of the main factors other than the lower fitness contributes to the formation of smaller plaque phenotype. Together, the reduced fitness and smaller plaque size suggested that the rF257C mutant could be attenuated.

The rF257C exhibits reduced virulence in suckling mice. Previous studies have shown that genetic diversity and replication error rates are linked to RNA virus pathogenesis, with alterations in viral polymerase fidelity leading to virus attenuation *in vivo* (8, 23, 35, 37, 42). Thus, the fitness deficit and smaller plaque size of the high-fidelity mutant rF257C lead us to further assess its virulence *in vivo*. Infections in suckling mice with a dose of 0.1 $TCID_{50}$ WT virus resulted in lethality of 4 out of 5 mice, whereas none died in the rF257C-inoculated group inoculated with the same dose (Fig. 5A). Increasing the dose by 1 $TCID_{50}$ resulted in full lethality when infected with WT virus by day 3, but not a single mouse infected with the rF257C mutant succumbed to infection (Fig. 5B). Only at the highest dose of 10 $TCID_{50}$ did we observe lethality by the mutant virus (3 out of 5 mice), albeit with slower kinetics relative to those of WT virus (Fig. 5C). No mice died in the control group inoculated with phosphate-buffered saline (PBS) (data not shown). We calculated the 50% lethal dose (LD_{50}) based on the $TCID_{50}$; the LD_{50} of the WT and the rF257C mutant are 0.04 $TCID_{50}$/100 μl and 6.9 $TCID_{50}$/100 μl, respectively. These results showed that the rF257C mutant exhibited a 172-fold reduction in virulence compared with that of the WT virus, indicating that the high-polymerase-fidelity variant rF257C is attenuated in suckling mice.

rF257C exhibits reduced virulence and transmissibility in pigs. To monitor the virulence of rF257C in a natural FMDV host, we used a porcine model of infection according to a previous protocol (43). The aim of the challenge test in pigs was to qualitatively determine whether the rF257C is attenuated. A dose of 10^5 $TCID_{50}$/pig of WT virus was selected as previously described (44), while a 10-fold higher dose was chosen for rF257C than for the WT based on experiments in suckling mice. Three pigs were inoculated intradermally in the heel bulbs with a dose of either 10^5 $TCID_{50}$/pig of WT or 10^6 $TCID_{50}$/pig of rF257C. Additionally, two naive pigs were moved to the room where the pigs were inoculated with WT or rF257C at 24 hpi and housed together for 7 days to study host-to-host transmission.

In the pigs inoculated with WT virus (animals 3, 4, and 5), the disease was observed as early as 2 dpi and reached a high clinical score by 3 to 4 dpi, with levels of viremia correlating with the appearance of clinical signs (Fig. 6A). However, only mild clinical

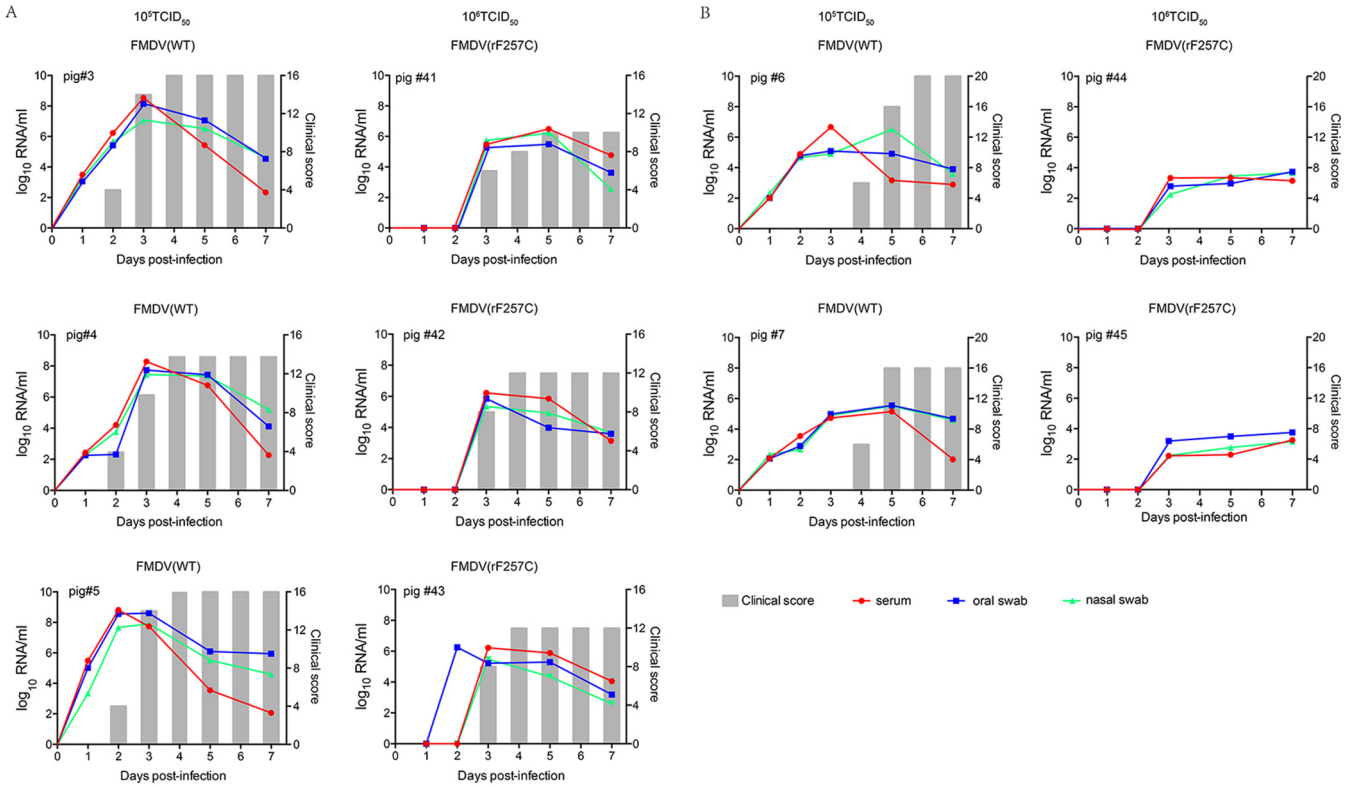

FIG 6 The rF257C variant is attenuated in pigs. Detection of FMDV RNA by reverse transcription-quantitative PCR (RT-qPCR) in nasal/oral swabs and serums collected from pigs infected with10^5 TCID$_{50}$ of FMDV WT or 10^6 TCID$_{50}$ of rF257C (A) and from their contact pigs (B). Time (in days postinoculation) is shown on the *x* axis, RNA copy numbers are shown on the left-hand *y* axis, and lesion scores are shown on the right-hand *y* axis.

signs were observed in pigs inoculated with a 10-fold higher dose of the rF257C (animals 41, 42, and 43) than that of the WT (Fig. 6A). Additionally, pigs inoculated with the rF257C mutant exhibited an approximately 100-fold reduction in viral RNA levels compared with those of pigs inoculated with WT virus (Fig. 6A). These data show that FMDV rF257C is attenuated in pigs compared to the WT, even when the inoculum is 10-fold higher than the parental virus.

For the contact-exposed pigs in the WT-inoculated group (animals 6 and 7), the viral RNA in blood and swabs was detected as early as 1 dpi and peaked at 2 to 3 dpi. The clinical signs were detectable at 4 dpi and reached a high clinical score between 5 to 6 dpi. Interestingly, no clinical signs occurred in the two contact pigs in the rF257C-inoculated group (animals 44 and 45), and the RNA copies produced from contact-exposed pigs showed a 100- to 1,000-fold reduction compared to that of the WT-inoculated group (Fig. 6B). These results show that transmissibility of the rF257C mutant virus, which exhibits increased polymerase fidelity, is significantly reduced in a natural host model *in vivo*.

DISCUSSION

We have previously shown that FMDV RdRp mutants with either increased or decreased fidelity are attenuated in suckling mice (22–24). However, the RdRp modifications in one serotype of FMDV can have very different effects on RdRp fidelity in a different serotype (24). It has been reported that a single amino acid change can affect the polymerase fidelity of RNA viruses (26–28, 31). Therefore, we mutated the highly conserved residue F257 located in the finger domain of the FMDV RdRp, and the F-to-C substitution resulted in a high-fidelity RdRp variant. A one-step growth curve revealed that while the growth kinetics of rF257C were similar to those of the parental virus (Fig. 2B), this mutant showed reduced fitness and produced smaller plaque in cells compared to the WT (Fig. 4B). Additionally, the rF257C mutant was attenuated both in

suckling mice and in pigs (Fig. 5 and 6), along with reduced transmissibility in pigs. Taken together, we show that polymerase fidelity is a determinant of FMDV virulence and transmissibility in a natural host.

Previous studies have demonstrated that RdRp fidelity is determined by a complex network of interactions and remote site mutations may alter the structural elements and motifs, including residues at the active site. The side chain of Phe257, along with the residues Phe244, Leu278, Met296, Pro297, Ile306, and Leu310, forms part of a highly hydrophobic cavity. It is possible that the residues within the hydrophobic cavity could affect polymerase fidelity by changing the conformation and altering the interactions with the incoming NTP. In addition, the residues Asn307 (motif B) and Asp245 (motif A) play an important role in nucleotide recognition (32). The residue F257 substitution could affect the nucleotide recognition by Asn307 and Asp24. Therefore, it is tempting to hypothesize that the Phe257 remote from the active site modulates polymerase fidelity via a conformational change in polymerase structure.

A better understanding of virus transmissibility is essential for FMD control. Our previous study showed that the ribavirin-selected FMDV mutants with high fidelity RdRp displayed attenuated phenotype in suckling mice (23). Here, a high-fidelity polymerase variant presented an attenuated phenotype not only in suckling mice but also in the pig natural host. Transmission studies in pigs demonstrated that the rF257C-inoculated group showed no clinical signs, and the viral RNA copy number derived from these contact-exposed pigs was 100- to 1,000-fold lower than that of the contact-exposed pigs in the WT-inoculated group (Fig. 6), indicating diminished transmissibility of FMDV with low genetic diversity. The lower genetic diversity and higher fidelity in rF257C could lead to slowing down the rate of evolution, thus reducing overall fitness and virulence of FMDV. During RNA virus replication, the high mutation rates are the by-product of selection for increased replicative speed (45), while the decreased host-to-host spread of rF257C is likely associated with slower viral replication speed *in vivo* (Fig. 6). This study provides evidence that FMDV transmissibility is significantly limited by reduced genetic diversity.

The worldwide distribution of serotype O FMDV stains could be due to their high virulence and transmissibility induced by high genetic diversity. Previously, we have shown that the mutation frequency of type O FMDV was 8.77 mutations/10^4 nucleotides, while that of the type Asia1 FMDV was only 4.26 mutations/10^4 nucleotides (23, 24). Higher genetic diversity of type O FMDV could increase both virulence and transmissibility, thus leading to the wider prevalence of type O FMDV. Although control measures were applied to international borders, the PanAsia strain has still spread across most of Asia into Europe and South Africa (19). In addition, multiple FMD outbreaks in many countries and regions have been caused by type O FMDV, including outbreaks in Taiwan in 1997 (46), the United Kingdom in 2001 and 2007 (47, 48), and Japan in 2001 and 2010 (49, 50), and multiple outbreaks in the Republic of Korea in 2010 and 2011 (51). For type O FMDV, virulence and transmissibility are different among different strains. In Japan, the 2000 outbreak was limited to four cattle farms, whereas the 2010 outbreak spread to 292 farms and resulted in the slaughter of approximately 300,000 animals (49, 50). A recent study demonstrated that the O/JPN/2000 strain had higher polymerase fidelity than the O/JPN/2010 strain (52), which may explain why O/JPN/2010 is a pandemic strain in Japan. Taken together, we emphasize that the inherently high genetic diversity of type O FMDV strains could be the cause of the prevalence of this serotype worldwide.

The current inactivated whole virus vaccine to control FMD has been effective in reducing the number of outbreaks worldwide. However, there is still a potential risk of the virulent virus escaping from vaccine manufacturing facilities, as FMDV is infectious at low doses and is capable of rapid transmission within susceptible animals (53). Moreover, FMDV could spread rapidly throughout a wide region and invade countries that were previously free from the disease. Engineering polymerase fidelity variants of viruses is a promising approach for the rational design of

TABLE 1 Primers used in this study

Primer	Sequence (5′–3′)	Usage
3D-F257C-U	GAT GCA ATG AAC ATC ATG TGC GAG GAG GTG TTC AAC	Generation of the rF257C mutant
3D-F257C-L	CGT GTT GAA CAC CTC CTC GCA CAT GAT GTT CAT TGC	Generation of the rF257C mutant
Frequency-U	CTG ACC CCG CCT ACG GGA AAG TG	Mutation frequency assay
Frequency-L	AGT AGT AAG TGG CAG TAC GGA GG	Mutation frequency assay
Fitness-U	AGG ACC GGT GAA GAA ACC TGT CG	Fitness assay
Fitness-L	AAA TAG GAA GCG GGA AAA GCC	Fitness assay

live-attenuated vaccines. As such, attenuated viruses with reduced transmissibility as seed viruses for the inactivated FMD vaccine production should be considered. A recent study showed that introduction of high-fidelity mutations generated a genetically more stable live-attenuated oral poliovirus vaccine strain (54). Given that the F257 of RdRp is highly conserved among all serotype strains of FMDV, engineering of the FMDV polymerase combined with other attenuation strategies could contribute to the development of a safer, genetically stable live-attenuated virus with reduced transmissibility.

In conclusion, we generated a single amino acid substitution FMDV variant with a high-fidelity polymerase associated with viral fitness, virulence, and transmissibility in a natural host. The attenuation and impaired transmissibility seen in this high-fidelity polymerase variant of FMDV highlights the fact that the maintenance of viral population diversity is essential for interhost viral spread.

MATERIALS AND METHODS

Ethics statement. All animal experiments were performed in enhanced biosafety level 3 (P3+) containment facilities approved by the Review Board of the Harbin Veterinary Research Institute (HVRI), Chinese Academy of Agricultural Sciences (CAAS). This study was carried out in strict accordance with the Chinese Regulations of Laboratory Animals—The Guidelines for the Care of Laboratory Animals (Ministry of Science and Technology of People's Republic of China) and the Laboratory Animal Requirements of Environment and Housing Facilities (GB 14925-2010; National Laboratory Animal Standardization Technical Committee). Protocols for the animal studies were approved by the Committee on the Ethics of Animal Experiments of the HVRI, CAAS (protocol number 170312-05 for mice and 171029-09 for pigs).

Cells and viruses. BHK-21 (baby hamster kidney cell line) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., South Logan, UT) and 1% penicillin-streptomycin at 37°C in 5% CO_2. The FMDV O/YS/CHA/05 strain of FMDV serotype O (GenBank accession number HM008917) used in our study was generated from the infectious cDNA clone pYS (55).

Molecular graphics. To model the structure of FMDV O/YS/CHA/05 RdRp ($3D^{pol}$), the amino acid sequences of $3D^{pol}$ were searched for homologies in the Protein Data Bank (PDB). The crystal structure of template 3KLV (PDB identifier 3KLV) was retrieved from the PDB and used for homology modeling. The protein modeling module Discovery Studio (DS) v4.5 (Accelrys, Inc.) was used to construct a three-dimensional (3D) model of the FMDV O/YS/CHA/05 polymerase. and the best models were selected on the basis of the analysis results of the internal scoring functions of the protein modeling module and the Profile-3D program. The chosen model was subjected to energy minimization to obtain a stable, low-energy conformation. PyMOL (http://www.pymol.org/) was used to locate the amino acid substitutions on the structure of the O/YS/CHA/05 $3D^{pol}$.

Construction of recombinant plasmids. Plasmid FMDV (clone pYS) was digested with EcoRI and EcoRV (TaKaRa, Dalian, China) and ligated using T4 ligase (New England BioLabs, Ipswich, MA) into a pOK12 vector that had been previously digested with the same restriction endonucleases. Subsequently, the plasmid pFMDV-3D, containing the $3D^{pol}$ coding region, and the plasmid pOK12 were digested with EcoRI/MluI (TaKaRa, Dalian, China) to generate pFMDV-3D (E/M). The resulting plasmid containing the $3D^{pol}$ gene was used as the template for site-directed mutagenesis using primers listed in Table 1. Positive plasmids bearing the desired mutations in $3D^{pol}$ were digested with EcoRI/MluI and reintroduced into the pYS infectious clone. The recombinant plasmids were used for *in vitro* transcription and transfection.

***In vitro* transcription and transfection.** The plasmids were linearized by digestion with EcoRV, and transcripts were generated using the RiboMax large-scale RNA production systems T7 kit (Promega). After transcription, the reaction mixture was treated with 1 U of RQI DNase/μg RNA (Promega). BHK-21 cells were transfected with 5 to 10 μg of *in vitro*-transcribed RNA using Effectene transfection reagent (Qiagen). The supernatant of the transfected cells was used to infect fresh monolayer BHK-21 cells. After 48 h of incubation at 37°C, viruses were harvested via three freeze-thaw cycles. The recovered viruses were passaged 10 times into BHK-21 cells, and the stability of the introduced mutations was confirmed by sequencing of the $3D^{pol}$-coding region.

$TCID_{50}$ assay and growth curve. Ten-fold serial dilutions of viruses were prepared in 96-well round-bottomed plates in DMEM. Dilutions were performed in octuplicate, and 50 μl of the dilution was transferred to 10^4 BHK-21 cells plated in 100 μl of DMEM with 2% FBS. After 3 days, $TCID_{50}$ values were determined by the Reed-Muench formula (56).

To determine viral replication kinetics, growth experiments in BHK-21 and IBRS-2 cells were performed as follows. First, cell monolayers in 6-well tissue culture plates were washed with phosphate-buffered saline (PBS) and inoculated with the different viruses at a multiplicity of infection (MOI; PFU number/cell) of 0.01. The plates were incubated for 1 h at 37°C, washed three times with PBS to remove unbound virus particles, and overlaid with DMEM supplemented with 2% FBS. The infected cells were incubated at 37°C and harvested at different times. The plates were subjected to three consecutive freeze-thaw cycles, and cell debris were removed by centrifugation. The viral titers of the supernatants were determined by $TCID_{50}$ assay. Mean values and standard deviations were calculated from three independent experiments.

Sequencing for mutational frequency. FMDV RNA was extracted using a Simply P total RNA extraction kit (BioFlux, Hangzhou, China), and cDNA was generated by reverse transcription of total RNA using PrimeScript reverse transcriptase (TaKaRa, Dalian, China). To determine mutation frequencies, a portion of the P1 gene region was amplified by PCR with the Easy-A high-fidelity PCR cloning enzyme (Stratagene, Foster City, CA), using the primers listed in Table 1. The PCR product was purified and cloned into the pMD18-T vector (TaKaRa, Dalian, China) for sequencing and analyzed using the Lasergene software package (DNAstar, Inc., Madison, WI). The number of mutations per 10^4 nucleotides (nt) sequenced was determined as the total number of mutations identified in each population divided by the total number of nucleotides sequenced for that population multiplied by 10^4. For each population, 60 to 80 partial P1 structural gene sequences of approximately 500 nt per replicate (primers flanking genome positions 2800 to 3300) were sequenced. Mutation frequencies (mutations per 10,000 nt) were determined as described previously (57).

RNA mutagen assay. Monolayers of BHK-21 cells were pretreated with various concentrations of ribavirin or 5-FU (Sigma, USA) for 3 h. These mutagen concentrations were not highly toxic to cells over the 72 h of incubation period. A concentration of 5-FU higher than 1,500 μM is cytotoxic to BHK-21 cells, while a 5-FU concentration lower than 750 μM has no effect on FMDV replication. Thus, 750 μM, 1,000 μM, and 1,500 μM were used to evaluate whether the FMDV high-fidelity mutant is resistant to 5-FU. Similarly, 25 to 200 μM ribavirin was used. The cells were infected with the rescued FMDV variants at an MOI of 0.01 for 1 h and were subsequently treated with the same mutagen concentration as during the pretreatment. The infected cell cultures were then incubated at 37°C in 5% CO_2 for 72 h. For the final stage of the experiment, viruses were released from the cells by three cycles of freeze-thaw, and the lysate was clarified by centrifugation at 6,500 $\times$ *g* for 5 min. The titer of the lysate was determined using a $TCID_{50}$ assay on BHK-21 cells. Mean values and standard deviations were calculated from three independent experiments.

Direct competition fitness assay. For the direct competition fitness assay, each of the rescued FMDV mutants was mixed with its parental virus O/YS/CHA/05 at a ratio of 9:1, 1:1, or 1:9 to infect BHK-21 cells in triplicate wells at an MOI of 0.1 over three passages. Viral RNA was extracted, and the region corresponding to the $3D^{pol}$ gene was amplified by reverse transcription-PCR (RT-PCR) for sequencing. The abundance of each competitor was measured as the height of the peak for the nucleotide corresponding to the WT or mutant sequence in the sequencing chromatogram.

Pathogenicity test of FMDV in suckling mice. Three-day-old BALB/c suckling mice were assigned to six groups (6 to 9 mice per group) and were inoculated cervicodorsally with 100 μl of diluted virus (0.1 to 10 $TCID_{50}$) in a 10-fold dilution series as previously described (22, 58, 59). The percent survival of the animals was recorded every 12 h for 7 days after inoculation.

Pathogenicity test of FMDV in pigs. Prior to infection, the pigs were assigned to two groups with three pigs per group, and housed in two separate rooms. One group was inoculated intradermally at the front right heel bulb with a dose of 10^5 $TCID_{50}$ for WT, and the other group was inoculated with a dose of 10^6 $TCID_{50}$ for rF257C. To investigate virus transmissibility, two contact-exposed pigs were comingled with infected donor pigs 24 h after inoculation. Blood and nasal/oral secretions were collected daily for up to 7 dpi. All animals underwent clinical evaluation, and rectal temperature was monitored daily throughout the experiment. Clinical scoring was performed based on the presence of vesicles in the mouth and on the feet; a maximum lesion score of 16 was possible in directly inoculated pigs (the inoculated foot was not counted in the determinations), and a score of 20 was possible for direct-contact pigs. FMDV RNA in plasma and swab samples was measured by real-time RT-PCR.

Statistical analysis. The mutation frequency was evaluated using a two-tailed paired *t* test. Growth curves and the RNA synthesis profiles of each mutant and FMDV WT were compared using repeated-measures ANOVA. Drug resistance was assessed using Student's *t* test. All statistical tests were conducted using GraphPad Prism software. *P* values of >0.05 were not significant (NS).

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