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Foot-and-mouth disease virus type O specific mutations determine RNA-dependent RNA polymerase fidelity and virus attenuation

Chen Li[a,1], **Haiwei Wang**[a,1], **Tiangang Yuan**[a], **Andrew Woodman**[b], **Decheng Yang**[a], **Guohui Zhou**[a], **Craig E. Cameron**[b], and **Li Yu**[a,*]

[a]Division of Livestock Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No. 678 Haping Road, Xiangfang District, Harbin 150069, PR China

[b]Department of Biochemistry & Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA

Abstract

Previous studies have shown that the FMDV Asia1/YS/CHA/05 high-fidelity mutagen-resistant variants are attenuated (Zeng et al., 2014). Here, we introduced the same single or multiple-amino-acid substitutions responsible for increased $3D^{pol}$ fidelity of type Asia1 FMDV into the type O FMDV O/YS/CHA/05 infectious clone. The rescued viruses O-DA and O-DAMM are lower replication fidelity mutants and showed an attenuated phenotype. These results demonstrated that the same amino acid substitution of $3D^{pol}$ in different serotypes of FMDV strains had different effects on viral fidelity. In addition, nucleoside analogues were used to select high-fidelity mutagen-resistant type O FMDV variants. The rescued mutagen-resistant type O FMDV high-fidelity variants exhibited significantly attenuated fitness and a reduced virulence phenotype. These results have important implications for understanding the molecular mechanism of FMDV evolution and pathogenicity, especially in developing a safer modified live-attenuated vaccine against FMDV.

Keywords

Foot-and-mouth disease virus; Polymerase; Fidelity; Fitness; Nucleoside analogue; Virulence

1. Introduction

Foot-and-mouth disease (FMD) is an acute, highly contagious disease affecting domestic and wild cloven-hoofed animals. Foot-and-mouth disease virus (FMDV), which belongs to the type species of the *Aphthovirus* genus within the *Picornaviridae* family, is the causative agent of FMD (Mason et al., 2003; Sobrino et al., 2001). The virus exists in the form of seven different serotypes: A, O, C, Asia1, and South African Territories 1 (SAT1), SAT2 and

*Correspondence to: Harbin Veterinary Research Institute, Harbin 150069, PR China. liyu@hvri.ac.cn (L. Yu).

[1]These authors contributed equally to this work.

SAT3, with each serotype containing multiple genotypes (Knowles and Samuel, 2003; Mason et al., 2003). FMDV is a single-stranded, positive-sense RNA genome of approximately 8500 nucleotides surrounded by four structural proteins to form an icosahedral capsid. The genome organization is similar to that of other picorna viruses, having a large single open reading frame (ORF) flanked by highly structured 5′ and 3′ untranslated regions (NTR). The ORF is translated into a single polyprotein that is processed by the three viral proteases Lpro, 2A, and 3C into the protein products P1 (VP4, VP2, VP3 and VP1), P2 (2A, 2B and 2C) and P3 (3A, 3B, 3Cpro and $3D^{pol}$) (Domingo et al., 2003; Grubman and Baxt, 2004).

FMDV replicate with extremely high mutation rates due to its error prone RNA-dependent RNA polymerase (RdRp) that lacks proofreading ability (Domingo et al., 1996; Domingo and Holland, 1997). And the mutant spectra that are generated upon replication of the virus called quasispecies (Andino and Domingo, 2015). It is widely accepted that low-fidelity replication is largely responsible for the capacity of RNA viruses population to evolve rapidly and overcome bottlenecks during intra-host transmission, and changing environments (Domingo, 2009; Duffy et al., 2008; Sanjuan, 2012). Indeed, modulation of RNA virus polymerase fidelity is a tool that can be used to attenuate an RNA virus population. Studies have shown that nucleoside analogues such as ribavirin or 5-fluorouracil (5-FU) can be misincorporated into viral genomes during RNA synthesis leading to error catastrophe (Airaksinen et al., 2003; Crotty et al., 2001). Passaging virus populations in the presence of mutagenic compounds has been shown to select for resistant strains that carry specific amino acid substitutions in their RdRp that increases their fidelity (Beaucourt et al., 2011), such as PV-G64S, CHIKV-C483Y, FMDV-R84H and WNV-V793I/G806R, the increase in RdRp fidelity for most of these variants leads to reduced fitness *in vitro* and attenuated virulence *in vivo* (Coffey et al., 2011; Pfeiffer and Kirkegaard, 2003; Van Slyke et al., 2015; Vignuzzi et al., 2008; Zeng et al., 2013). One explanation for the decreased fitness is that diversity in the RNA virus population is reduced, meaning fewer variants that contains potentially advantageous adaptive mutations are present (Perales et al., 2007). More recently, low-fidelity mutator RdRp variants of PV, FMDV and CVB3 have been constructed through point mutation. This increases mutation frequency beyond the natural state of a virus, which also leads to an attenuated phenotype *in vivo* (Gnadig et al., 2012; Korboukh et al., 2014; Xie et al., 2014). These results have demonstrated that a reduction in replication fidelity is also an available approach to modulate the virulence of an RNA virus population (Gnadig et al., 2012; Liu et al., 2013; Pfeiffer and Kirkegaard, 2005; Rai et al., 2017; Xie et al., 2014).

Clearly, an increase or decrease in RdRp fidelity is a mechanism that can be used to attenuate the virus and be used as an antiviral strategy. We have previously demonstrated that increase or decrease in RdRp fidelity can attenuate the type Asia1 FMDV (Xie et al., 2014; Zeng et al., 2014). In this study, we introduced the same RdRp amino acid substitutions that are responsible for increased type Asia1 FMDV polymerase fidelity into the type O FMDV. We were able to rescue variants and unexpectedly show that O-DA and O-DAMM have lower polymerase fidelity than that of the wild-type (WT) type O virus. Using selection in the presence of ribavirin and 5-FU we were able to isolate novel type O FMDV high-fidelity RdRp mutants, termed AMMR and T64I.

Our results showed that the O-DAMM and AMMR mutants replicated *in vitro* at near wild-type levels, but were significantly attenuated *in vivo*. Our analysis on the fidelity and virulence of these variants allows us to conclude that higher replication fidelity changes are associated with a more attenuated virus population and that multiple amino acid substitutions in RdRp determine the virulence of type O FMDV. Importantly, these results also revealed that FMDV RdRp modifications in one serotype have very different effects on fidelity in another serotype and should be considered in the context of rational vaccine development.

2. Materials and methods

2.1. Cells and viruses

BHK-21 (baby hamster kidney cell line), 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. O/YS/CHA/05 (GenBank accession number: HM008917), the WT strain FMDV O/YS/CHA/05 of FMDV serotype O used in our study, was generated from the infectious cDNA clone pYS (Yang, 2009).

2.2. Construction of recombinant plasmids

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

2.3. In vitro transcription and transfection

The plasmids were linearized by digestion with *Eco*RV, 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 5–10 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 ten times into BHK-21 cells, and the stability of the introduced mutations was confirmed by sequencing of the $3D^{pol}$-coding region.

2.4. $TCID_{50}$ assay and growth curve

Tenfold serial dilutions of virus were prepared in 96-well round-bottom 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 (Reed and Muench, 1938).

To determine viral replication kinetics, growth experiments in BHK-21 cells were performed as follows. First, cell monolayers in 6-well tissue culture plates were washed with phosphate-buffered saline (PBS) and inoculated with different viruses at a multiplicity of infection (MOI, pfu number/cell) of 0.01. The plates were incubated for 1 h at 37 °C. Then, the cells were washed three times with PBS to remove unbound virus particles and covered 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 was 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.

2.5. 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 part of the P1 structural gene 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. The sequencing data was analyzed using the Lasergene software package (DNASTAR Inc., Madison, WI). The number of mutations per 10^4 nucleotides 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, 60–80 partial P1 structural gene sequences of approximately 500 nt per replicate (primers flanking genome positions 2800–3300) were sequenced. Mutation frequencies (mutations per 10,000 nt) were determined as described previously (Beaucourt et al., 2011).

2.6. RNA mutagen assays

BHK-21 cell monolayers were pretreated with various concentrations of ribavirin or 5-FU (Sigma, USA) for 3 h. These mutagen concentrations were not highly toxic to the cells over the 72 h incubation period (Supplemental Fig. S1). 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, virus was released from the cells by three cycles of freeze-thaw, and the lysate was clarified by centrifugation at 6500×*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.

2.7. 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 RT-PCR for sequencing (Table 1). 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.

2.8. Determination of virulence in suckling mice

BALB/c suckling mice were purchased from the Harbin Veterinary Research Institute, CAAS, China. The animal experiments were approved by the Animal Ethics Committee of the Harbin Veterinary Research Institute, CAAS, China. Three-day-old BALB/c suckling mice were assigned to 18 groups (6–9 mice per group) and were inoculated cervicodorsally with 100 μL of diluted virus (0.1–100 $TCID_{50}$) in a 10-fold dilution series, as previously described (Baranowski et al., 2003; Gutierrez-Rivas et al., 2008; Zeng et al., 2013). The percent survival of the animals was recorded every 12 h for 7 d after inoculation.

2.9. 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>0.05 were not significant (NS).

3. Results

3.1. Generation of the type O FMDV mutants carrying the type Asia1 high-fidelity amino acid substitutions

We introduced amino acid substitution(s) identified in the Asia type1 serotype in order to understand the impact of the same mutation (s) on polymerase fidelity in the type O serotype of FMDV. In total of four different mutations, D5N, A38V, M194I and M296V, were introduced into the $3D^{pol}$ coding region of the type O FMDV to produce single amino acid substitution D5N, A38V, and two amino acid substitutions D5N and A38V, as well as four amino acid substitutions D5N, A38V, M194I and M296V using FMDV O/YS/CHA/05 infectious clone. BHK-21 cells were transfected with the corresponding *in vitro*-transcribed RNA genomes. All constructs produced infectious virion, which were designated as O-D5N, O-A38V, O-DA and O-DAMM (Fig. 1A). Each virus was the passaged independently to determine the genetic stability of each mutant. After ten passages, the rescued virus was detected by RT-PCR, sequencing results showed the $3D^{pol}$ mutations in the virus mutants were stable with no secondary site compensatory mutations in $3D^{pol}$ region (data not shown); however, T135I substitution in 2C was simultaneously appeared in the virus mutants and the wide-type virus, which could improve FMDV *in vitro* replication (Yuan et al., 2017) but has no effect on 3Dpol fidelity. Quantification of each virus population by $TCID_{50}$ showed similar yields for each variant to WT (Fig. 1B).

3.2. The type O FMDV mutants O-DA and O-DAMM with type Asia1 3D substitutions are low-fidelity variants

As the four RdRp variants had been characterized as high fidelity in the type Asia1 strain we examined the replication fidelity of the rescued mutants in the context of a type O strain. For each viral population at passage 10, a 500 bp fragment of the capsid protein-encoding region

from 60 to 80 individual clones was sequenced and used to determine the average number of mutations per 10^4 nt. All of the rescued mutants exhibited different mutation frequencies compared to WT, which presented 8.77 mutations/10^4nt sequenced: 9.72 mutations for O-D5N variant (P = ns), 8.45 mutations for O-A38V variant (P = ns), 10.07 mutations for O-DA variant (P<0.05 by a two-tailed paired *t*-test), and 13.14 mutations for O-DAMM variant (P<0.01 by a two-tailed paired *t*-test) (Fig. 2A). Indeed, our data showed that the O-DA and O-DAMM mutants produced significantly more mutations than that of the FMDV WT, with fidelity reduced by 1.15- and 1.50-fold, respectively (Fig. 2A).

To exclude the possibility that the increased mutation frequency of the variants was the result of enhanced replication capacity and to study their function in the viral lifecycle, we performed growth curve to address concern. In BHK-21 cells, no significant differences in the production of infectious particles were observed between each mutant and the FMDV WT (Fig. 2B). But at the early time of infection, the production of infectious particles of O-D5N, O-DA and O-DAMM were lower than that of FMDV WT. Together, this observation indicated that the low-fidelity observed for these variants was not caused by the enhancement in virus replication.

To evaluate the impact of reduced RdRp fidelity on the tolerance to mutagens, the sensitivity of each mutant to nucleoside analogues (ribavirin and 5-FU) was tested (Fig. 2CD). The results showed that the O-D5N, O-A38V, O-DA and O-DAMM mutants exhibited a significantly reduced yield when exposed to 200 μM concentrations of ribavirin compared to WT (Fig. 2C). Additionally, the rescued mutant viruses were treated with two different concentrations of 5-FU. At the higher 1500 μM 5-fluorouracil concentration, the O-A38V, O-DA and O-DAMM mutants displayed a significant reduction in virus yield to that of the WT, but for O-D5N, no statistically significant difference (P = ns) was observed (Fig. 2D). Together, these results showed that a reduction in replication fidelity of the four rescued FMDV polymerase mutants resulted in their increased sensitivity to the mutagen treatment with an overall fall in virus yield.

3.3. Generation of the FMDV populations resistant to ribavirin and 5-fluorouracil

To select type O FMDV high-fidelity variants, we passaged FMDV in the ribavirin and 5-FU-containing medium as described previously (Beaucourt et al., 2011). WT FMDV rescued from the infectious clone pYS was serially passaged 22 times in the presence or absence of 50 μM ribavirin or 800 μM 5-FU. To identify the mutation(s) conferring resistance to ribavirin or 5-FU, the $3D^{pol}$ gene was amplified from the ribavirin and 5-FU selected 18th passage population and sequenced. The amino acid substitutions A122T, M194I, M296I and R440W appeared in the ribavirin-selected viral population, while T64I and D133N appeared in the 5-FU-selected viral population. No additional mutations were found from p18 to p22 in the $3D^{pol}$-coding region of these FMDV mutants. These observations strongly suggested that one or more of these substitutions mediated resistance to the nucleoside analogues. We subsequently introduced the identified mutations into the type O infectious clone individually, or in combination. All variants were infectious and were designated as A122T, R440W, T64I, D133N and AMMR.

In most cases, resistance to a mutagen(s) is associated with increased polymerase fidelity (Coffey et al., 2011; Levi et al., 2010; Vignuzzi et al., 2008). To examine the replication fidelity of our RdRp mutants, their mutation frequency was determined in the absence of mutagens. All of the rescued mutants exhibited different mutation frequencies compared with the WT. Our data showed that the AMMR and T64I mutants were indeed high fidelity variants that produced significantly fewer mutations than did WT ($P<0.01$ by a two-tailed paired *t*-test), with 1.40- and 1.29-fold increases in fidelity, respectively (Fig. 3A). To exclude the possibility that the reduced mutation frequency in the AMMR and T64I mutants resulted from weakened viral replication, we examined the replication kinetics of the rescued FMDV polymerase mutant viruses. The kinetics of virus replication of each variant was compared to the WT virus, with no significant differences in the production of infectious particles being observed between each mutant and the WT (Fig. 3B). We concluded that the higher fidelity observed for the AMMR and T64I mutants were not caused by any replication defects.

To investigate the resistance level of the high fidelity AMMR and T64I mutants, we exposed replicating virus to higher doses of ribavirin and 5-FU and then quantified the production of infectious virus (Fig. 3CD). At a concentration of 1500 μM 5-FU, only the T64I showed a significant increase in resistance, while the AMMR variant showed no significant difference. Besides both variants showed resistance to increasing 5-FU concentrations (Fig. 3C). Both variants displayed a significant increase in the ribavirin resistance ratio compared to that of the parental virus at a concentration of 200 μM ribavirin (Fig. 3D). These results indicate that increased polymerase fidelity is associated with increased resistance to ribavirin and partial resistance to higher doses of 5-FU.

3.4. The FMDV fidelity variants exhibit reduced in vitro fitness

RNA viruses are associated with a high RdRp error rate. This produces the genetically diverse population following replication that allows the virus to adapt to a variety of selective pressures. We hypothesized that high-fidelity variants would have a replicate disadvantage as a result. To test our hypothesis *in vitro*, we performed direct competition assays with WT virus using our identified high-fidelity variants. The specific codon at the corresponding RdRp position was used to differentiate the mutants from the WT under various doses of WT-v-Mutant (Fig. 4). To ensure correct ratio of WT-to-mutant virus inoculation, RNA was extracted and subjected to RT-PCR analysis. The chromatogram peaks corresponding to the O-D5N, O-DA, O-DAMM, AMMR and T64I mutations clearly show a mixed population that represented the inoculating dose prior to pass aging (top panels for each variant, Fig. 4). After 3 passages in BHK-21 cells, the same chromatogram peaks corresponding to the O-D5N, O-DA, O-DAMM, AMMR and T64I mutations were non-detectable (Fig. 4). These results confirm that the O-D5N, O-DA, O-DAMM, AMMR and T64I mutants have an obvious fitness disadvantage over the WT in BHK-21 cells, which we associate with their increased fidelity phenotype.

3.5. The O-DAMM and AMMR are attenuated in suckling mice

To evaluate the effects of increased or decreased fidelity on virulence of FMDV, we chose the well-established 3-days suckling mice as animal model (Rodriguez-Pulido et al., 2011;

Zeng et al., 2014). After inoculation with a dose of 1 $TCID_{50}$, all mice died in the WT, O-D5N, O-A38V and T64I mutant inoculated groups, but no mice died in the AMMR mutant inoculated groups (Fig. 5B and D), four out of six mice died in the O-DAMM mutant inoculated group and five out of six mice died in the O-DA mutant inoculated group (Fig. 5B). At the highest dose 10 $TCID_{50}$, all mice in the AMMR mutant inoculated group died (Fig. 5E). No mice died in the control group inoculated with PBS. Our results showed that the AMMR, O-DAMM and O-DA exhibited 40-, 20-, and 10-fold viral attenuation in suckling mice, respectively (Fig. 5). By contrast, the single amino acid mutant T64I and O-D5N exhibited no reduction in virulence. Together, these data suggested multiple amino acid substitutions in the RdRp can attenuate the virulence of type O FMDV.

4. Discussion

FMD is known to be one of the most extremely contagious viral diseases of cloven-hoofed animals. Many laboratories have therefore focused their research on discovering the virulence factors that are associated with its causative agent, FMDV. For example, the well-established role of the leader proteinase in the virulence in of swine and cattle (Kleina and Grubman, 1992; Roberts and Belsham, 1995; Skern et al., 1998), the role of RGD sequence in the VP1 G-H loop (Leippert et al., 1997; Mason et al., 1994), and the importance of virus nonstructural proteins (Gladue et al., 2014; Pacheco et al., 2013, 2003; Rai et al., 2017), the 3′ NTR (Rodriguez Pulido et al., 2009) and the codon usage bias (Diaz-San Segundo et al., 2015). We have previously shown that increased $3D^{pol}$ fidelity of the type Asia1 FMDV strain could reduce its fitness *in vitro* and attenuate virulence *in vivo* (Zeng et al., 2014).

The manipulation of RdRp fidelity has been long been proposed as a mechanism to attenuate an RNA virus, for example a SARS-CoVExoN mutator strain has been suggested as a vaccine candidate. The researchers in this example demonstrated that the mutator strain had decreased fidelity *in vivo,* which resulted in attenuation in young, aged, and immunocompromised mouse models of human SARS (Graham et al., 2012). With regard to FMDV, it has been shown that either increased or decreased RdRp fidelity attenuates virus growth in animals (Rai et al., 2017; Zeng et al., 2014). Therefore, manipulation of polymerase fidelity is clearly a promising approach to engineer attenuated virus vaccines.

Our initial intention in this study was to engineer high-fidelity variants of FMDV by manipulating relatively conserved sites in the polymerase. We introduced known high-fidelity variants of the Asia1 strain into the type O strain. Our results unexpectedly showed that the rescued viable FMDV polymerase variants exhibited decreased or unchanged rather than increased replication fidelity. This was evident by the inability of the O-D5N, O-A38V, O-DA and O-DAMM mutants to tolerate the 5-FU and ribavirin mutagens as compared to WT (Fig. 2CD). This was further supported by the observations that show that the low-fidelity RdRp mutants generated more genomic diversity (Fig. 2A). The use of nucleoside analogues allowed us to isolate and characterize novel type O mutants with decreased sensitivity to the mutagens (Fig. 3). Two of the selected viruses showed significantly lower genetic diversity that did not impact the replication kinetics when compared to WT virus (Fig. 3AB). In both examples, modulation of fidelity impacted fitness *in vitro*, as all variants were out-competed by WT virus following serial passage (Fig. 4).

The results observed in our *in vivo* study strongly suggested that manipulation of RdRp fidelity can attenuate the virus (Fig. 5). Further, our results indicate that multiple amino acid substitutions in RdRp determine the virulence of type O FMDV rather than single amino acid substitutions (Fig. 5). The high-fidelity AMMR and low-fidelity O-DAMM were attenuated 40- and 20-fold respectively, whereas single variants showed no attenuation.

It is interesting to note that, the same mutation in different serotypes of FMDV have diametrically opposite effects on replication fidelity. There are 15-amino acid differences between type Asia1 FMDVAsia1/YS/CHA/05 and type O FMDV O/YS/CHA/05 in the $3D^{pol}$ region. It is possible that the observed replication fidelity differences between type Asia1 and type O was caused by these amino acid differences. For type C FMDV, growth in the presence of ribavirin results in the sequential selection of three amino acid substitutions (M296I, P44S and P169S) (Agudo et al., 2010). For the type Asia1 FMDV it was four different amino acid substitutions (D5N, A38V, M194I, M296V) in the viral polymerase ($3D^{pol}$) (Zeng et al., 2014). These results strongly suggest that the amino acid substitutions that determine the fidelity of the polymerase may be very different in distinct FMDV serotypes and that the same amino acid substitutions may have opposing effects on virus fidelity and virulence.

We cannot exclude additional contributing factors to diversity. For example, it has been shown that two mechanisms (RdRp-dependent *versus* RdRp-independent) contribute to the genetic diversity and the mutation frequency of the PV-H273R population in HeLa cells (Korboukh et al., 2014). In addition, a single amino acid mutation in the WNV methyltransferase, T248I, is associated with decreased fidelity (Van Slyke et al., 2015). So potentially the low-fidelity of O-DAMM and O-DA may not only be due to the characteristics of the polymerase. Eight geographically distinct genetic lineages (topotypes) have been identified for the type O FMDV, that differ by approximately 15% at the nucleotide level (Samuel and Knowles, 2001). Therefore, the attenuated phenotype of type O FMDV lineages will probably be determined by multiple amino acid substitutions instead of single amino acid substitution in the RdRp due to the inherent virus variation that exists between each lineage. Besides, for FMDV, there are 17-amino acid difference between Asia1/YS/CHA/05 and O/YS/CHA/05 in $3D^{pol}$ region. It may change the 3Dpol interact with other proteins such as 2C which can also modulate nucleotide incorporation (Agudo et al., 2016).

The genetic diversity of an RNA virus population is closely related to its pathogenicity (Coffey and Vignuzzi, 2011). Previous studies have shown that poliovirus carrying a high-fidelity polymerase replicates at wild type levels, generated less genomic diversity and is unable to adapt to adverse growth conditions in infected animals. The reduced viral diversity led to loss if neurotropism and an attenuated pathogenic phenotype (Korboukh et al., 2014; Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006). In this study, we use FMDV as model, the multiple amino acid mutants O-DAMM and AMMR replication fidelity changed 1.55-fold and 1.40 fold- compared to WT, resulting in attenuated virulence in suckling mice. The fidelity variants provide a panel of viruses producing populations with genetic diversity both higher and lower than WT. Both mutants are attenuated in an animal model, suggesting that the change of FMDV replication fidelity has a marked effect on viral adaptation and

pathogenicity. Our lab has constructed a series of FMDV attenuated strains using other route and we found that the trend of virus attenuation in the sucking mice model and natural host is identical although the degree of attenuation quantity is different (unpublished data, inpreparation), which showed that attenuation of FMDV in the sucking mice model does imply attenuation in natural hosts. Besides, fidelity-altering mutations associated with single or few residues, combining these with other conventional and effective attenuating mutations or methods may be a good strategy to further reduce reversion to virulence as live attenuated vaccines (LAVs) (Borderia et al., 2016).

We explored the modulation of FMDV polymerase fidelity and its correlation with fitness and virulence. The results provide key insights into the role of replication fidelity in the evolutionary dynamics of RNA viruses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2018.01.030.

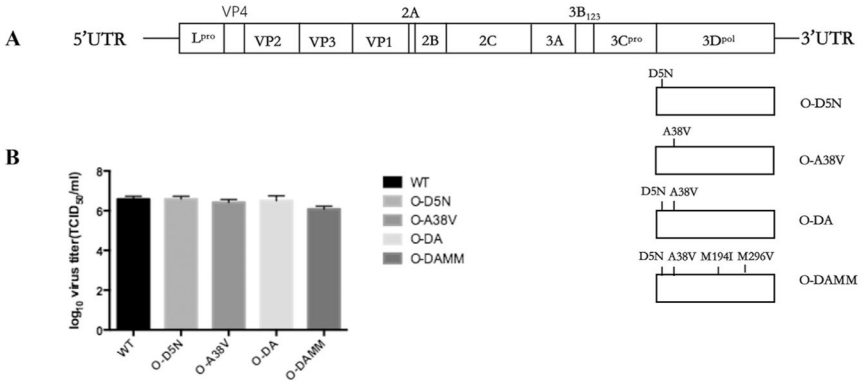

Fig. 1.

Generation of the type O FMDV 3D mutants with the Asia1 high-fidelity amino acid substitution. (A) Schematic of the FMDV RNA genome and the mutant sites of $3D^{pol}$. (B)The replication ability of FMDV 3D mutants in BHK-21 cells. The mutants and their parent virus (WT) were passaged ten times in BHK-21 cells, and the titers of progeny viruses were determined by a $TCID_{50}$ assay. The standard deviations (n = 3) of mean virus titers are shown.

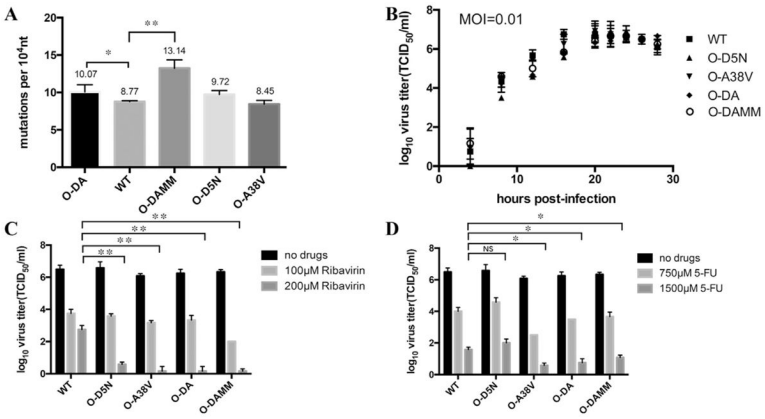

Fig. 2.

The type O FMDV 3D mutants with Asia1 3D substitutions are low-fidelity variants. (A) A mean of seventy partial P1 sequences (approximately 35,000 nucleotides per replicate) were obtained. The mean mutation frequencies (number of nucleotide mutations per 10,000 nt sequenced)±SD represent the averages of all the replicates. The same pattern of reduced mutation frequency for other polymerase mutants compared to the WT was observed for each replicate (two-tailed Mann-Whitney test, n = 3, *P<0.05, **P<0.01). (B) Replication ability and of FMDV RdRp low-fidelity mutants. BHK-21 cells were infected with the mutants O-D5N, O-A38V, O-DA, O-DAMM or FMDV WT at an MOI of 0.01. The virus harvested at different times were titrated and expressed as $TCID_{50}$. Mean values±SD are shown (repeated-measures ANOVA, n = 3; ns for all mutants compared with WT). (C and D) RNA virus mutagen sensitivity assay of viral strains. BHK-21 cells, treated with different concentrations of RNA mutagen or mock-treated, were infected at MOI of 0.01; 72 h post-infection, the infectivity in the progeny virus was determined by $TCID_{50}$ assay. Mean virus titers ±SD are shown (Student's *t*-test, n = 4; *P<0.05, **P<0.01).

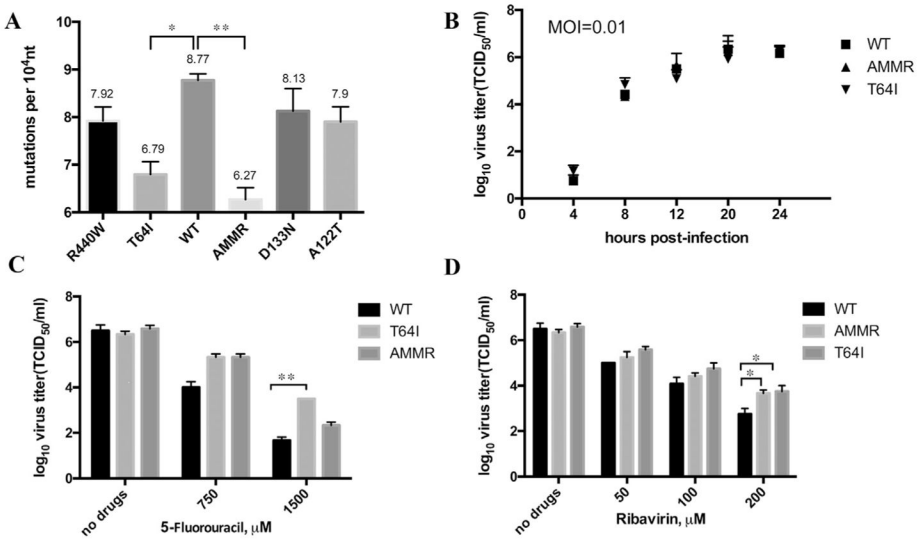

Fig. 3.

Generation of the FMDV populations resistant to ribavirin and 5-FU. (A) A mean of seventy partial P1 sequences (approximately 35,000 nucleotides per replicate) were obtained. The mean mutation frequencies (number of nucleotide mutations per 10,000 nt sequenced)±SD represent the averages of all the replicates. The same pattern of reduced mutation frequency for other polymerase mutants compared to the WT was observed for each replicate (two-tailed Mann-Whitney test, n =3, *P<0.05, **P<0.01). (B) Replication ability of the FMDV RdRp high-fidelity mutants T64I and AMMR. BHK-21 cells were infected with the mutants or FMDV WT at an MOI of 0.01. Viruses harvested at different times were titrated and expressed as $TCID_{50}$. Mean values ±SD are shown (repeated-measures ANOVA, n = 3; ns for all mutants compared with WT). (C and D) RNA virus mutagen sensitivity assay of viral strains. BHK-21 cells, treated with different concentrations of RNA mutagen or mock-treated, were infected at MOI of 0.01; 72 h post-infection, the infectivity in the progeny virus was determined by $TCID_{50}$ assay. Mean virus titers±SD are shown (Student's *t*-test, n = 4; *P<0.05, **P<0.01).

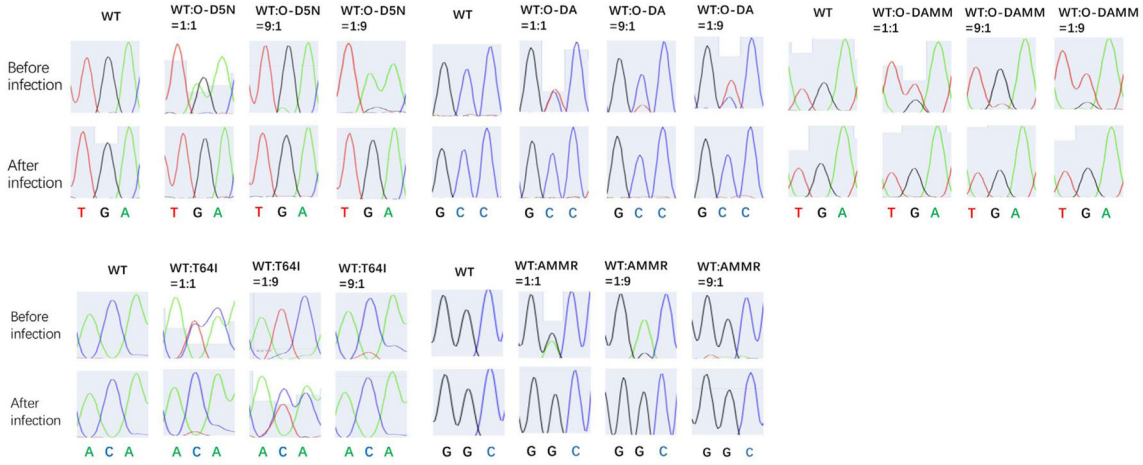

Fig. 4.

The FMDV fidelity variants exhibit reduced fitness *in vitro*. The mutant O-D5N, O-DA, O-DAMM, T64I and AMMR was mixed with the WT at a ratio of 9:1, 1:1 or 1:9 to inoculate BHK-21 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 (A or T nt) or the WT (G or C nt) in sequencing chromatograms.

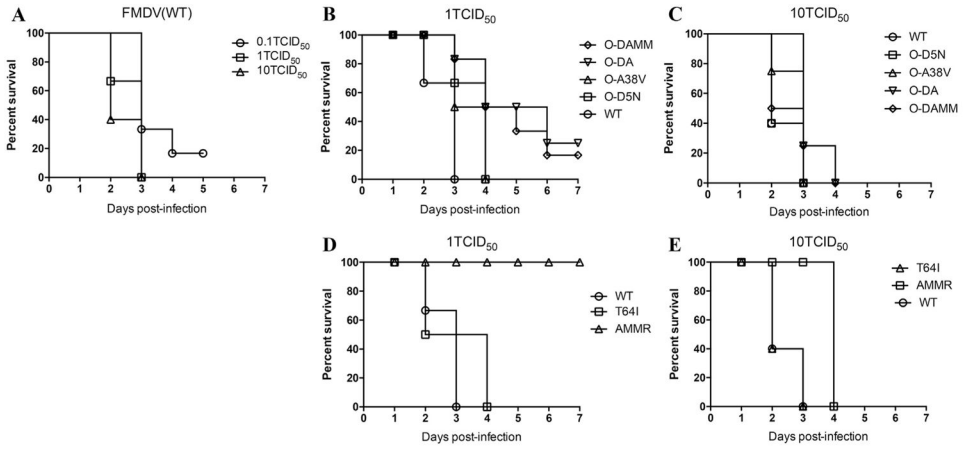

Fig. 5.

The O-DAMM and AMMR are attenuated in 3-day old BALB/c suckling mice. A total of 15 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 $TCID_{50}$, 1 $TCID_{50}$ (B and D) or 10 $TCID_{50}$ (C and E). Animal deaths were scored for up to 7 days after inoculation, and survivors were euthanized ($n = 6–9$ for each group).

Table 1

Primers used in this study.

Primers	Sequences (5′-3′)	Usage
Fitness-U	AGGACCGGTGAAGAAACCTGTCG	For 3tness assay
Fitness-L	AAATAGGAAGCGGGAAAAGCC	
Frequency-U	CTGACCCCGCCTACGGGAAAGTG	For mutation frequency assay
Frequency-L	AGTAGTAAGTGGCAGTACGGAGG	
3D-D5N-U	CGAGGGATTGATAGTTAACACCAGAGATGTTGAG	Production of the D5N mutant
3D-D5N-L	CTCAACATCTCTGGTGTTAACTATCAATCCCTCG	
3D-A38V-U	GAATTTGGGCCTGCCGTCTTGTCCAACCAG	Production of the A38V mutant
3D-A38V-L	GTCCTGGTTGGACAAGACGGCAGGCCCAAATTC	
3D-M194I-U	CATTCTTTACACCAGGATTATGATTGGTAGATTTT	Production of the M194I mutant
3D-M194I-L	GCAAAATCTACCAATCATAATCCTGGTGTAAAGAA	
3D-M296V-U	CACTGTTGAAGGCGGGGTGCCGTCTGGTTGTT	Production of the M296V mutant
3D-M296V-L	GAACAACCAGACGGCACCCCGCCTTCAACA GTG	
3D-A122T-U	CTGGCCTCCCCTGGACCCTCCAGGGGAAACGCC	Production of the A122T mutant
3D-A122T-L	GTTTCCCCTGGAGGGTCCAGGGGAGGCCAGGCG	
3D-M296I-U	CTGTTGAAGGCGGGATACCGTCTGGTTGTTCCGCG	Production of the M296I mutant
3D-M296I-L	GAACAACCAGACGGTATCCCGCCTTCAACAGTG	
3D-R440W-U	GACCTGATGAGTACTGGCGTCTCTTTGAGCCTTTC	Production of the R440W mutant
3D-R440W-L	AAAGGCTCAAAGAGACGCCAGTACTCATCAGGTCC	
3D-T64I-U	TAAACACAAGGAAAACATCAAGATGTCTGAGGAGG	Production of the T64I mutant
3D-T64I-L	CTCCTCAGACATCTTGATGTTTTCCTTGTGTTTAG	
3D-D133N-U	CCGTGGTGCGCTCATTAACTTCGAGAACGGCACTG	Production of the D133N mutant
3D-D133N-L	GACAGTGCCGTTCTCGAAGTTAATGAGCGCACCAC	