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Ribavirin-Resistant Variants of Foot-and-Mouth Disease Virus: the Effect of Restricted Quasispecies Diversity on Viral Virulence

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ABSTRACT

Mutagenic nucleoside analogues can be used to isolate RNA virus high-fidelity RNA-dependent RNA polymerase (RdRp) variants, the majority of which are attenuated *in vivo*. However, attenuated foot-and-mouth disease virus (FMDV) high-fidelity RdRp variants have not been isolated, and the correlations between RdRp fidelity and virulence remain unclear. Here, the mutagen ribavirin was used to select a ribavirin-resistant population of FMDV, and 4 amino acid substitutions (D5N, A38V, M194I, and M296V) were identified in the RdRp-coding region of the population. Through single or combined mutagenesis using a reverse genetics system, we generated direct experimental evidence that the rescued D5N, A38V, and DAMM mutants but not the M194I and M296V mutants are high-fidelity RdRp variants. Mutagen resistance assays revealed that the higher replication fidelity was associated with higher-level resistance to ribavirin. In addition, significantly attenuated fitness and virulence phenotypes were observed for the D5N, A38V, and DAMM mutants. Based on a systematic quantitative analysis of fidelity and virulence, we concluded that higher replication fidelity is associated with a more attenuated virus. These data suggest that the resulting restricted quasispecies diversity compromises the adaptability and virulence of an RNA virus population. The modulation of replication fidelity to attenuate virulence may represent a general strategy for the rational design of new types of live, attenuated vaccine strains.

IMPORTANCE

The ribavirin-isolated poliovirus (PV) RdRp G64S variant, the polymerases of which were of high replication fidelity, was attenuated *in vivo*. It has been proposed (M. Vignuzzi, E. Wendt, and R. Andino, Nat. Med. 14:154–161, http://dx.doi.org/10.1038/nm1726) that modulation of replication fidelity is a promising approach for engineering attenuated virus vaccines. The subsequently mutagen-isolated RdRp variants also expressed the high-fidelity polymerase, but not all of them were attenuated. Few studies have shown the exact correlation between fidelity and virulence. The present study investigates the effect of restricted quasispecies diversity on viral virulence via several attenuated FMDV high-fidelity RdRp variants. Our findings may aid in the rational design of a new type of vaccine strain.

Foot-and-mouth disease (FMD) is a widespread, epizootic, transboundary animal disease affecting cloven-hoofed wildlife and livestock (1–4). The significant economic losses resulting from FMD are due to the high rates of morbidity of infected animals and stringent trade restrictions imposed on affected countries. Foot-and-mouth disease virus (FMDV), an *Apthovirus* within the *Picornaviridae* family, is a single-strand, positive-sense RNA virus and the etiological agent of FMD. There are 7 serotypes of FMDV (serotypes O, A, C, and Asia1 and Southern African Territories [SAT] serotypes SAT-1, SAT-2, and SAT-3) (5, 6). The infectious virion is a nonenveloped icosahedron composed of 4 structural proteins (VP1, VP2, VP3, and VP4). The approximately 8,400-nucleotide (nt) genome contains a single open reading frame (ORF), which is translated into a polyprotein that is processed by the 3 viral proteases Lpro, 2A, and 3C into the polypeptide products P1 (VP1 to VP4), P2 (2A, 2B, and 2C), and P3 (3A, 3B, 3Cpro, and 3Dpol). Further cleavage of these regions yields 14 mature virus proteins and several protein intermediates that are required for viral replication (7, 8).

Many viruses, including hepatitis C virus, poliovirus (PV), influenza virus, and FMDV, have RNA genomes. These viruses replicate with extremely high mutation rates due to the lack of proofreading by their RNA-dependent RNA polymerases (RdRps); as a result, their significant genetic diversity allows them to rapidly adapt to dynamic environments and to evolve resistance to antiviral drugs. Mutagenic nucleoside analogues such as ribavirin, 5-fluorouracil (5-FU), and 5-azacytidine (AZC) can be incorporated into the viral genome during viral RNA synthesis, resulting in a significant increase in the frequency of deleterious mutations due to mispairing in subsequent replication cycles (9–13). The increased mutation frequency places a greater-than-usual number of genomic RNAs beyond a hypothetical error threshold. Accordingly, RNA virus populations without sufficient error repair mechanisms lie close to this threshold, and even moderate increases in mutation frequency have the potential to severely diminish infectivity and to generate lethal mutations (14–18). The resulting lethal mutations have been exploited to develop efficacious antiviral strategies that rely on viral replication errors (13, 19), as revealed by PV and other RNA viruses (16, 20–22). Interestingly, mutagens delivered at appropriate concentrations that do not give rise to large-scale lethal mutagenesis in the viral genome can be used

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to isolate mutagen-resistant RdRp variants, some of which are characterized by significantly increased polymerase fidelity, as verified by sequence analysis and/or biochemical assays (23–26). Using this strategy, high-fidelity RdRp mutants, including the PV G64S mutant, the coxsackievirus group B3 (CVB3) A372V mutant, and the chikungunya virus (CHIKV) C483Y mutant, have been successfully isolated (23, 24, 26). However, the amino acids determining RdRp fidelity differ in RNA viruses.

PV G64S and CHIKV C483Y mutants have decreased fitness *in vitro* and attenuated virulence *in vivo* (23, 27), while the virulence phenotype of the CVB3 A372V mutant has not been determined (24). In addition, the directly constructed PV RdRp K359R mutant, which exhibited higher fidelity than the G64S mutant, is also attenuated (28). One explanation for this reduced fitness is that a diverse RNA virus population contains, by chance, more variants with potentially advantageous adaptive mutations, whereas a less diverse population is not as likely to possess such variants (23). Thus, the attenuated phenotype of these mutants is the result of the restricted quasispecies diversity (29). High-fidelity FMDV RdRp mutants isolated by mutagen selection have not been reported (25, 30). We recently isolated the FMDV high-fidelity R84H mutant using 5-FU, but unfortunately, this mutant did not exhibit an attenuated phenotype, possibly due to a moderate increase in replication fidelity (31). Obviously, these observations are not enough to clearly show the correlation between the increase in replication fidelity or the restriction in quasispecies diversity and viral phenotypes, including fitness and virulence. However, the modulation of polymerase fidelity remains a promising approach for engineering of attenuated virus vaccines (27).

In this study, we explored whether attenuated FMDV high-fidelity RdRp mutants could be isolated by using ribavirin. The FMDV RdRp mutants selected by ribavirin showed higher polymerase fidelity than the wild-type virus. For the first time, several FMDV high-fidelity RdRp mutants showed extremely decreased *in vitro* fitness and an attenuated phenotype in suckling mice. Importantly, our quantitative analysis of fidelity and virulence also clearly demonstrated that quasispecies diversity is a determinant of viral virulence within a viral population.

MATERIALS AND METHODS

Cells and viruses. BHK-21 (baby hamster kidney cell line), IBRS-2 (swine kidney cell line), primary bovine testis, primary bovine kidney, and bovine thymus cells were grown in Dulbecco modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories Inc., South Logan, UT) and 1% penicillin-streptomycin at 37°C in 5% CO_2. Asial/YS/CHA/05 (GenBank accession number GU931682), the wild-type (WT) strain of FMDV serotype Asia1 used in our study, was generated from the infectious cDNA clone pAsi (32).

Viral passage under mutagen selection pressure. The isolation of RdRp variants by passaging of FMDV with mutagen selection pressure was performed as previously described (33). Briefly, BHK-21 cell monolayers were pretreated with 50 μM ribavirin (Sigma, St. Louis, MO) for 2 h, incubated with FMDV for 1 h at a multiplicity of infection (MOI) of 0.05, and subsequently treated with 50 μM ribavirin. FMDV was harvested from the culture mixtures within 72 h postinfection (p.i.), and the progeny viruses were subjected to the next round of treatment for a total of 24 passages. The harvested virus stocks were sequenced at intervals of 4 passages until the 24th passage. Mean titers were determined by using a 50% tissue culture infective dose ($TCID_{50}$) assay in BHK-21 cells.

Construction of recombinant 3Dpol plasmids with the desired amino acid substitution(s). Plasmid pAsi, the infectious cDNA clone of Asia1 FMDV Asia1/YS/CHA/05, was digested with MluI and EcoRV or SmaI and MluI (TaKaRa, Dalian, China). The resulting fragment was ligated, using T4 DNA ligase (New England BioLabs, Ipswich, MA), into a pOK12 vector that had been previously digested with the same restriction endonucleases. The resulting plasmid containing the 3Dpol gene was used as the template for site-directed mutagenesis using the series of primers listed in Table 1. Positive plasmids bearing the desired mutation(s) in 3Dpol were digested with MluI and EcoRV or SmaI and MluI and reintroduced into pAsi that had been digested previously with the same restriction endonucleases. 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 by using the RiboMAX Large Scale RNA Production Systems-T7 kit (Promega, Madison, WI). After transcription, the reaction mixture was treated with 1 U RQI DNase/μg RNA (Promega, Madison, WI). BHK-21 cells were transfected with 5 to 10 μg of *in vitro*-transcribed RNA by using Effectene transfection reagent (Qiagen, Valencia, CA). The supernatants of the transfected cells were used to infect fresh BHK-21 cell monolayers. After a 48-h incubation at 37°C, viruses were harvested via 3 freeze-thaw cycles. The recovered viruses were passaged for 10 generations in BHK-21 cells, and the stability of the introduced mutations was confirmed by sequencing of the 3Dpol-coding region.

Determination of viral titers by a $TCID_{50}$ assay. Tenfold serial dilutions of virus were prepared in 96-well round-bottom plates in DMEM without FBS. 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 (34).

RNA mutagen assays. BHK-21 cell monolayers were pretreated with 1,200 μM 5-FU (Sigma, St. Louis, MO), 1,000 μM AZC (Sigma, St. Louis, MO), or 0 to 125 μM ribavirin for 2 h. These mutagen concentrations were not greatly toxic to the cells over the 72-h incubation period. The cells were infected with the rescued FMDV variants at an MOI of 0.01 or 1 for 1 h and were subsequently treated with the same concentration of mutagen as that used during the pretreatment. Within 72 h p.i., the virus was harvested by using 3 freeze-thaw cycles, and the infectious titer was determined by the $TCID_{50}$ assay. The mean values and standard deviations were calculated from 3 independent experiments.

One-step growth curve analysis of FMDV replication. To determine the replication kinetics of the rescued mutants in greater detail, one-step growth curves were performed via $TCID_{50}$ assays in both BHK-21 and IBRS-2 cells. Briefly, cells cultured in 6-well plates were inoculated with different viruses at an MOI of 10 and maintained at 37°C in 5% CO_2. The infected cell cultures were harvested at different time points by using 3 freeze-thaw cycles. Cell debris was removed by centrifugation, and the titers in the supernatants in 96-well plates were determined. The mean values and standard deviations were calculated from 3 independent experiments.

RNA synthesis during FMDV replication. To confirm FMDV genome synthesis during virus replication, we quantified FMDV genome copies by using a previously described real-time reverse transcription-PCR (RT-PCR) assay (35, 36). Briefly, total cellular RNA was extracted from FMDV-infected cells by using TRIzol (Invitrogen, Carlsbad, CA), and real-time PCR amplification was performed on a Stratagene Mx Real-Time qPCR system (Agilent Technologies) according to the manufacturer's instructions. The mean values and standard deviations were derived from triplicate measurements. The primers used to perform quantitative RT-PCRs are listed in Table 1.

Indirect and direct competition fitness assays. An indirect competition fitness assay was performed according to previously reported methods (23). In brief, each mutant and the WT virus, neither of which have a PstI restriction site at position 249 of RdRp, competed with a marked virus, a variant of the WT virus that contains a PstI restriction site. After 3 passages in BHK-21 or IBRS-2 cells at an MOI of 0.1, viral RNA was extracted from the culture supernatants, followed by reverse transcription and PCR using primers flanking position 249 of RdRp. Finally, a restric-

TABLE 1 Primers used in this study

Primer	Sequence (5′–3′)	Usage
3Dpol-F1	TTTCATCGTCGGCACTCACTC	Sequencing of the full-length 3Dpol-encoding gene
3Dpol-F2	TGCGCTGATTGACTTCGAGAAC	
3Dpol-R	GGAATGTGGAAGCGGGAAAAG	
D5N-mu-F	CGAGGGATTGGTTGTTAACACCAGAGATGTGGG	Production of the D5N mutant
D5N-mu-R	CTCCACATCTCTGGTGTTAACAACCAATCCCTCG	
A38V-mu-F	GAATTTGGGCCTGCTGTCTTGTCCAACAAGGAC	Production of the A38V mutant
A38V-mu-R	GTCCTTGTTGGACAAGACAGCAGGCCCAAATTC	
M194I-mu-F	CACATTCTTTACACCAGGATTATGATTGGCAGATTTTGGC	Production of the M194I mutant
M194I-mu-R	GCACAAAATCTGCCAATCATAATCCTGGTGTAAAGAATGTG	
M296V-mu-F	CATTGTTGTTGAGGGCGGGGTGCCGTCTGGCTGTTCCGC	Production of the M296V mutant
M296V-mu-R	GCGGAACAGCCAGACGGCACCCCGCCCTCAACAACAATG	
Real-time-F	AATGCACTCAAACAACGGAC	Quantitative real-time PCR for detecting RNA synthesis
Real-time-R	GCAGTGGTTAGCATCAAAGG	
Di-Fitness-F1	TTTCATCGTCGGCACTCACTC	Amplification of 3D for the direct fitness assay
Di-Fitness-F2	TGCGCTGATTGACTTCGAGAAC	
Indi-Fitness-F	CGCGCAAGAGACAGCAGATGG	Amplification of 3A to 3D for the indirect fitness assay
Indi-Fitness-R	GGAATGTGGAAGCGGGAAAAG	
Mu-frequency-F	TGTCCAACCTTCCTCCGCTTC	Amplification of VP3 for the mutation frequency assay

tion fragment length polymorphism assay was performed by using the PstI restriction enzyme; in this assay, a 2.5-kb fragment corresponded to the mutant or WT virus, while 1.8-kb and 0.7-kb fragments corresponded to the marked competitor. ImageJ software was used, and fitness was calculated as the output-to-input ratio of each mutant FMDV to the marked competitor.

For direct competition fitness assays, each mutant was mixed with the WT virus at a ratio of 9:1, 1:1, or 1:9 to infect BHK-21 or IBRS-2 cells in triplicate wells at an MOI of 0.1 over 3 passages. Viral RNA was extracted, and the region flanking RdRp amino acid 5 or 38 was amplified by RT-PCR for sequencing. The abundance of each competitor was measured as the height of the nucleotide encoding either the WT (nucleotide G or C) or each mutant (nucleotide A or T) in sequencing chromatograms. The primers used in the indirect and direct competition assays are listed in Table 1.

Sequencing. When all the cells showed cytopathic effect (CPE), viral RNA in the supernatants was extracted by using a Simply P Total RNA extraction kit (BioFlux, Hangzhou, China), and cDNA was generated by reverse transcription of total RNA by using PrimeScript reverse transcriptase (TaKaRa, Dalian, China). The entire 3Dpol sequence or 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 primer sequences listed in Table 1 (Fig. 1A). The PCR product was purified and cloned into the pMD18-T vector (TaKaRa, Dalian, China) for sequencing. The sequencing data were analyzed by using the Lasergene software package (DNAStar Inc., Madison, WI). To determine mutation frequencies, the number of mutations per 10^4 nucleotides sequenced was determined by dividing the total number of mutations identified in each population by the product of the total number of nucleotides sequenced for that population multiplied by 10^4. For each population, at least 65 partial P1 structural gene sequences of approximately 500 nt per replicate (primers flanking genome positions 2760 to 3260) were sequenced. The mutation frequencies (mutations per 10,000 nt) were determined as previously described (33). For further sequencing, 3 replicates of each virus were passaged 3 times in BHK-21 cells under the selection pressure of 100 μM ribavirin. The mutation frequency of each third-passage population under ribavirin pressure was determined by using the method described above.

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 to 9 mice per group) and were inoculated cervicodorsally with 100 μl of diluted virus (0.01 to 10 $TCID_{50}$) in a 10-fold dilution series, as previously described (37, 38). The percent survival of the animals was recorded every 12 h until 7 days after inoculation.

Replication ability of FMDV mutants in bovine-derived cells. Primary bovine testis cells, primary bovine kidney cells, and bovine thymus cells were continuously infected with diluted FMDV at 50 μl/well and incubated at 37°C in 96-well plates. At 3 days p.i., the viral titer per milliliter was determined by a $TCID_{50}$ assay. For RNA synthesis, the 3 cell types were incubated with the mutants at an MOI of 0.1 for 1 h in 12-well plates, and the cells were subsequently washed 3 times by using phosphate-buffered saline (PBS) and supplemented with DMEM containing 2% FBS. The infected cell cultures were harvested at different time points (12, 24, 36, and 48 h) after 3 freeze-thaw cycles. Cell debris was removed by centrifugation, and total cellular RNA was extracted by using TRIzol (Invitrogen). Real-time PCR amplification was performed on a Stratagene Mx Real-Time qPCR system (Agilent Technologies) according to the manufacturer's instructions. The mean values and standard deviations were derived from triplicate measurements.

Statistical analysis. The mutation frequency was evaluated by using the two-tailed Mann-Whitney U test. One-step growth curves and the RNA synthesis profiles of each mutant and WT FMDV were compared by using repeated-measures analysis of variance (ANOVA). Drug resistance was assessed by using Student's *t* test. All statistical tests were conducted with Graphpad Prism (Graphpad Software, San Diego, CA). *P* values of >0.05 were considered not significant (NS).

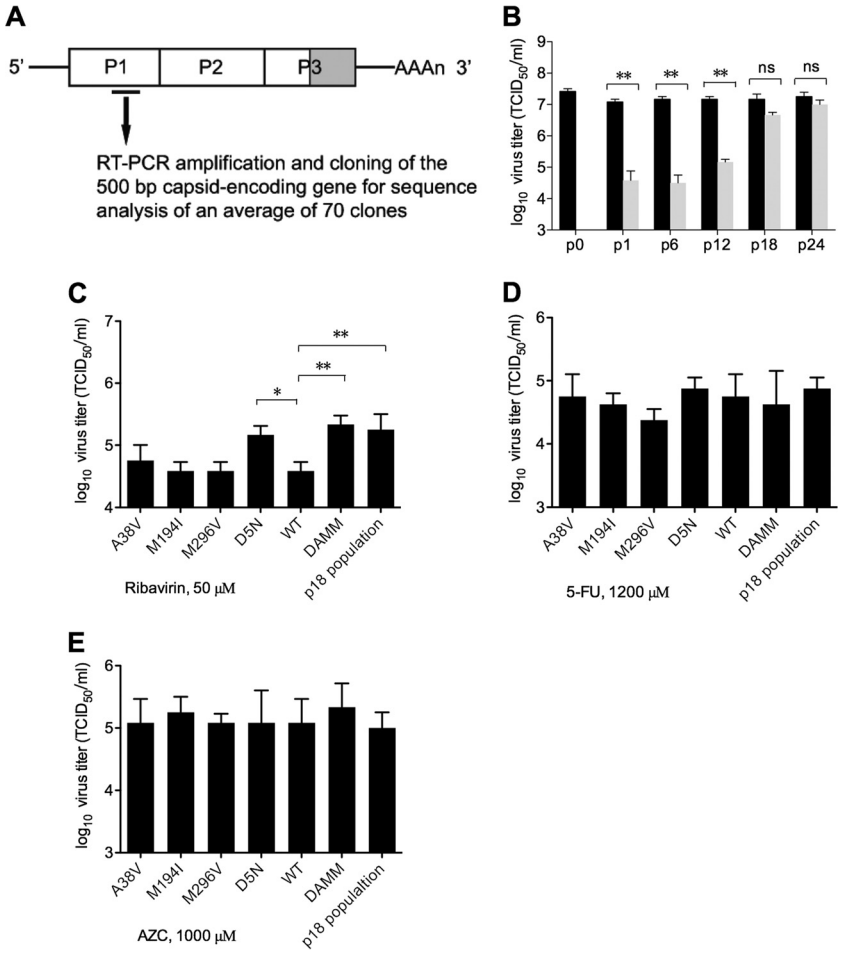

FIG 1 Isolation of ribavirin-resistant FMDV mutants. (A) Schematic of the FMDV RNA genome. The 3Dpol region is shaded in gray. A 500-bp section of the viral capsid gene was RT-PCR amplified and cloned for sequencing. Individual clones were used to determine the mutation frequencies presented throughout this study. (B) Generation of ribavirin-resistant FMDV mutants. The WT virus was serially passaged in the presence (gray) or absence (black) of 50 μM ribavirin at an MOI of 0.01. p0 is the initial WT stock titer. The mean titers of harvests ± standard deviations are shown ($n = 3$) (**, $P < 0.01$ by Student's t test). (C) Resistance of the D5N, A38V, and DAMM mutants to ribavirin. Matched titers of each mutant, the p18 population, or the WT were inoculated into triplicate wells with BHK-21 cells and 50 μM ribavirin. The mean titers ± standard deviations are shown ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$ [determined by Student's t test]). (D and E) None of the mutants were resistant to 5-FU (D) or AZC (E). BHK-21 cells pretreated with 1,200 μM 5-FU or 1,000 μM AZC were infected with each mutant; WT FMDV and the ribavirin-selected p18 population at an MOI of 0.01 were used as controls. Within 72 h after infection, the titer of progeny virus was determined by a $TCID_{50}$ assay using BHK-21 cells. The mean virus titers ± standard deviations are shown ($n = 3$) (no significant differences were identified by Student's t test [ns]).

RESULTS

Generation of FMDV populations resistant to ribavirin. To select FMDV high-fidelity variants, WT FMDV rescued from the infectious clone pAsi was serially passaged 24 times in the presence or absence of 50 μM ribavirin. After 1 passage (passage 1 [p1]), the mean titer of ribavirin-treated FMDV decreased by approximately 2.6 logs ($P < 0.01$) compared with untreated FMDV (Fig. 1B). By passage 18 (p18), no significant reduction in infectious titer was observed for ribavirin-treated FMDV compared with WT FMDV. In addition, this ribavirin-resistant phenotype was observed consistently from passage 18 to passage 24, indicating that a stable ribavirin-resistant viral population had been generated.

The D5N amino acid substitution confers resistance of FMDV to treatment with 50 μM ribavirin. To identify the mutation(s) conferring resistance to ribavirin, the 3Dpol gene was amplified from the ribavirin-selected 18th passage (the p18 population) and sequenced. The 3Dpol gene was chosen because polymerase mutations are usually the mediators of mutagen resistance in RNA viruses. The amino acid substitutions D5N, A38V, M194I, and M296V appeared in the ribavirin-selected 18th-passage viral population, and no additional mutations were found from p18 to p24 in the 3Dpol-coding region of these FMDV mutants. These results suggested that 1 or more of these 4 substitutions mediated resistance to ribavirin. To verify this hypothesis, the RdRp mutants with single-amino-acid substitutions were rescued and designated D5N, A38V, M194I, and M296V. To account for the possibility that these single-amino-acid substitutions have a synergistic effect on mutagen resistance and/or polymerase fidelity, a mutant carrying all 4 amino acid substitutions was rescued and designated DAMM. Subsequently, the rescued mutant viruses were treated with 50 μM ribavirin, the concentration used for the selection of the ribavirin-resistant FMDV populations. Similar to

the p18 population, the D5N and DAMM mutants exhibited increased resistance to ribavirin compared to WT FMDV ($P = 0.013$ and 0.002, respectively, by Student's t test). In contrast, no resistance to ribavirin was observed for the A38V, M194I, and M296V mutants (Fig. 1C). These results demonstrate that the D5N amino acid substitution in RdRp confers resistance to treatment with 50 μM ribavirin in FMDV.

To determine whether these mutants tolerated other nucleoside analogue mutagens, the D5N, A38V, M194I, M296V, and DAMM mutants were treated with 5-FU (1,200 μM) or AZC (1,000 μM). None of the mutants tolerated treatment with 5-FU or AZC (Fig. 1D and E).

The D5N, A38V, and DAMM mutants are high-fidelity RdRp variants. Previous studies of selected high-fidelity RdRp variants have consistently revealed that resistance to a mutagen(s) is associated with increased polymerase fidelity (23, 24, 27, 31). To examine the replication fidelity of our RdRp mutants, their mutation frequency was determined in the absence or presence of ribavirin.

The inherent fidelity of the RdRp mutants was first examined in the absence of ribavirin. For each viral population at passage 3, a 500-bp fragment of the capsid protein-encoding region from 65 to 72 individual clones was sequenced to determine the average number of mutations per 10^4 nt. The mutation frequencies of the rescued variants differed from that of the WT, which presented 4.26 mutations/10^4 nt sequenced: 2.82 mutations for the D5N variant ($P < 0.05$ by a two-tailed Mann-Whitney U test), 2.59 mutations for the A38V variant ($P < 0.05$), 2.27 mutations for the DAMM variant ($P < 0.01$), 4.02 mutations for the M194I variant, and 4.33 mutations for the M296V variant (P value was NS) (Fig. 2A). Thus, the D5N, A38V, and DAMM mutants produced significantly fewer mutations than did WT FMDV, with 1.51-, 1.65-, and 1.88-fold increases in fidelity, respectively.

To determine if the observed mutagen resistance was correlated with a lower mutational burden, we compared the mutation frequencies of each variant and WT FMDV after treatment with a high dose of ribavirin (100 μM). The mutational frequencies of each variant differed compared to that of WT FMDV, which generated 21.6 mutations/10^4 nt sequenced: 15.3 mutations for the D5N variant (substantially lower than that of the WT despite a NS P value), 14.2 mutations for the A38V variant ($P < 0.05$ by a two-tailed Mann-Whitney U test), 12.1 mutations for the DAMM variant ($P < 0.05$), 18.3 mutations for the M194I variant, and 20.7 mutations for the M296V variant (P values were NS) (Fig. 2B). These data revealed that the D5N, A38V, and DAMM mutants were higher-fidelity polymerase variants and that the tolerance of the D5N mutant to 50 μM ribavirin was associated with the enhancement of its polymerase fidelity.

To exclude the possibility that the reduced mutation frequency in the D5N, A38V, and DAMM mutants resulted from weakened viral replication, we examined the replication kinetics of the rescued FMDV polymerase mutant viruses. The kinetics of RNA synthesis and virus infectivity in each variant were compared with those of the WT virus. In BHK-21 and IBRS-2 cells, no significant differences in the production of infectious particles were observed between each mutant and the WT (Fig. 3A to D). In addition to infectious virus yield, we quantified total genomic RNA by real-time PCR. All polymerase mutants and the WT virus produced similar levels of RNA in both cell types (Fig. 3E to H). Thus, the higher fidelity observed for the D5N, A38V, and DAMM mutants was not caused by replication defects.

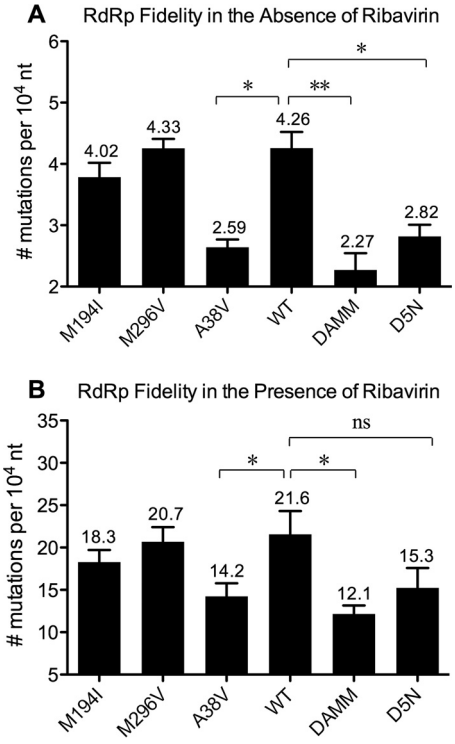

FIG 2 Mutation frequencies of each mutant and WT FMDV. Three independent stocks of virus were generated in ribavirin-free medium (A) and in medium containing 100 μM ribavirin (B). A mean of 70 partial P1 sequences (approximately 35,000 nucleotides per replicate) were obtained. The mean mutation frequencies (number of polymorphisms per 10,000 nt sequenced) ± standard deviations represent the averages of all replicates; the same pattern of reduced mutation frequency for each mutant compared to WT FMDV was observed for each replicate ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$ [determined by a two-tailed Mann-Whitney U test]).

The increase in polymerase fidelity determines the degree of viral mutagen resistance. Although the A38V mutant polymerase had increased replication fidelity, it was not resistant to 50 μM ribavirin, contradicting the hypothesis that enhanced polymerase fidelity confers resistance to mutagens (23, 24, 31). This led us to investigate the underlying mechanism of this unexpected phenomenon. The resistance of the mutants to higher ribavirin concentrations was evaluated by infection at a low MOI (0.01) in the presence of increasing concentrations of ribavirin to allow the cumulative effect of the increased mutation frequency to be observed over 2 or more replicative cycles. The A38V mutant tolerated treatment with increased ribavirin concentrations of 100 μM or higher ($P < 0.05$ or $P < 0.01$ by Student's t test) (Fig. 4A), while the M194I and M296V mutants showed sensitivity to ribavirin at different concentrations, similar to the WT (Fig. 4B and C). Importantly, the DAMM mutant was even more resistant to ribavirin than the A38V mutant at a concentration of >50 μM ($P < 0.01$ by Student's t test) (Fig. 4D). In contrast, the D5N mutant exhibited decreased resistance to increasing ribavirin concentrations ($P < 0.05$ by Student's t test) (Fig. 4E). These results indicate that increased polymerase fidelity is associated with increased mutagen resistance. Based on these observations, infections were performed with a high MOI. The D5N, A38V, and DAMM mutants

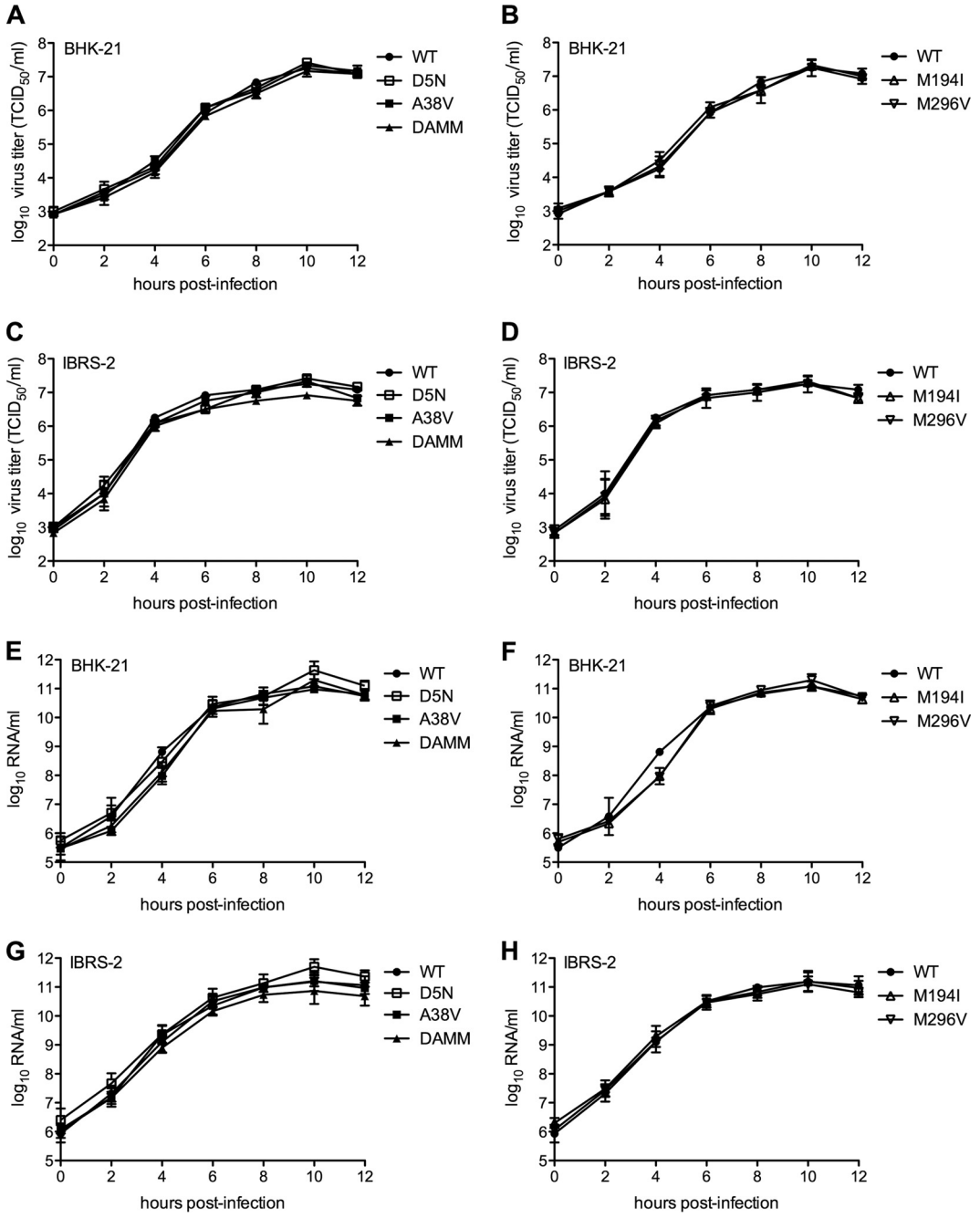

FIG 3 One-step growth curves and RNA synthesis of each mutant and the WT. BHK-21 and IBRS-2 cells were infected with mutant or WT virus at an MOI of 10. The resulting virus was harvested at different times, titers were determined and expressed as a $TCID_{50}$ dose (A to D), and the genome copy numbers were measured (E to H) for the same samples by real-time RT-PCR. The mean values ± standard deviations are shown ($n = 3$) (no significant differences were identified by repeated-measures ANOVA).

did not exhibit dramatically decreased sensitivity to the same concentration of ribavirin (Fig. 4F to H), consistent with the deleterious effect of mutations being reversed by population size, as predicted by population genetics theory (39–43). Taken together, these data indicate that the A38V mutant and, in particular, the DAMM mutant intrinsically possess high-level resistance to ribavirin.

The reduced genetic diversity of the mutant populations lowers *in vitro* fitness. In RNA viruses, a high replication error rate is required to quickly adapt to a variety of selective pressures. To assess whether these mutations incur a fitness cost, 2 types of competition assays were performed in 2 types of mammalian cells. In a more quantitative but indirect assay, fitness was determined by competing each virus against the same marked reference competitor under identical conditions. Both the D5N and A38V mutants exhibited significantly decreased fitness in BHK-21 and IBRS-2 cells, while the M194I and M296V mutants showed fitness similar to that of the WT (Fig. 5A and B). Moreover, the highest

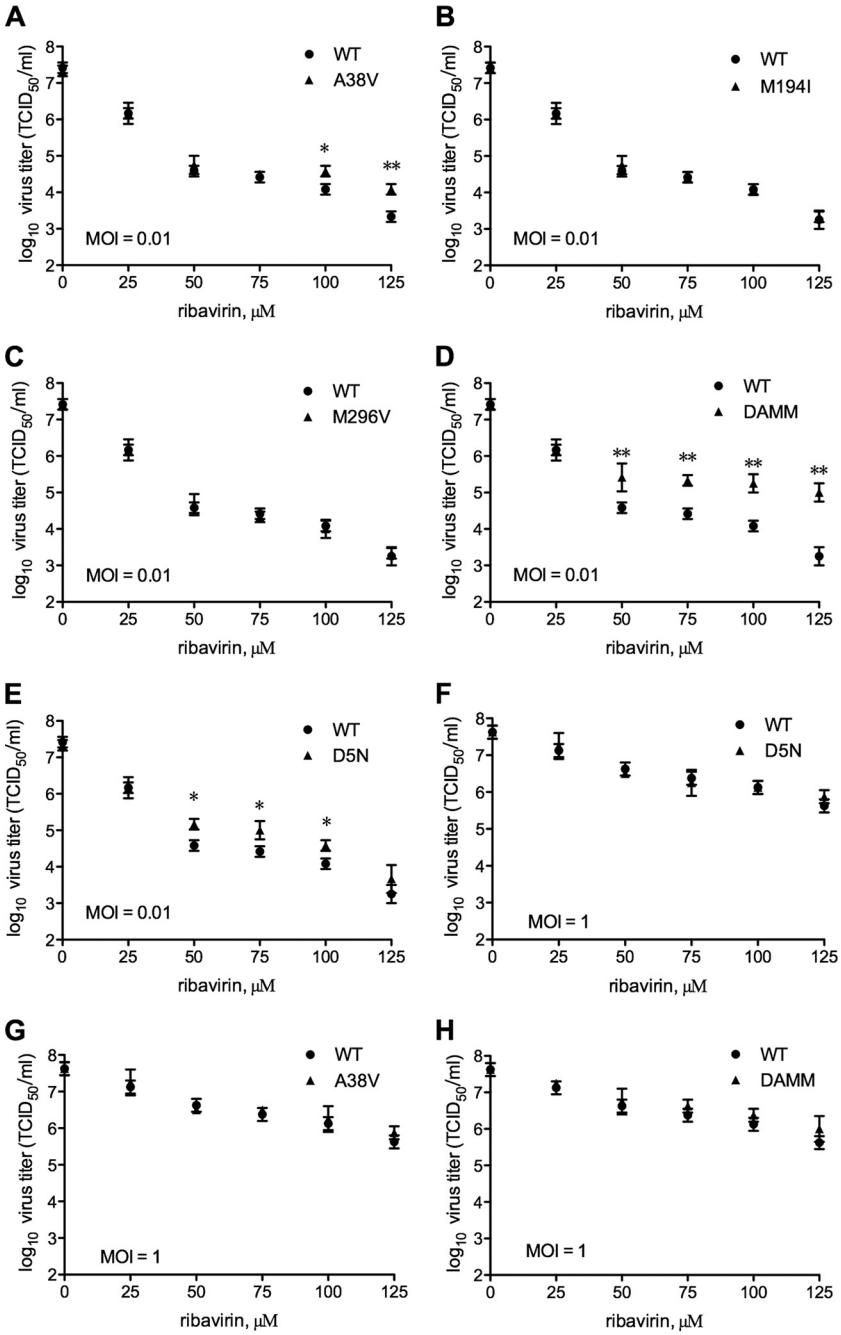

FIG 4 Mutagen resistance of each mutant. BHK-21 cells (2.5×10^6 cells in 6-well plates) were pretreated with ribavirin for 2 h prior to infection with each mutant or WT virus at an MOI of 0.01 (A to E) or 1 (F to H), as previously described (51). The cells were incubated with ribavirin at the indicated concentrations until all cells demonstrated CPEs, at which time the virus was collected and titers were determined by using BHK-21 monolayers. The mean titers ± standard deviations are shown ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$ [determined by Student's t test]).

reduction in fitness in both cell types was observed for the DAMM mutant (Fig. 5A and B). Taken together, these results indicate that the D5N, A38V, and DAMM mutants were significantly less fit than the WT in BHK-21 and IBRS-2 cells, even though the replication of these viruses was similar to that of the WT in both cell types.

To further examine the fitness landscape of the D5N, A38V, and DAMM mutants, we performed direct competition assays. The specific codon at the corresponding RdRp position was used to differentiate the mutants from the WT. After 3 passages in BHK-21 or IBRS-2 cells, the chromatogram peaks corresponding to the D5N, A38V, and DAMM mutations were extremely reduced, mirroring the data from the indirect competition assay (Fig. 5C). These results confirm that the D5N, A38V, and DAMM mutants have an obvious fitness disadvantage compared with the WT in both cell lines.

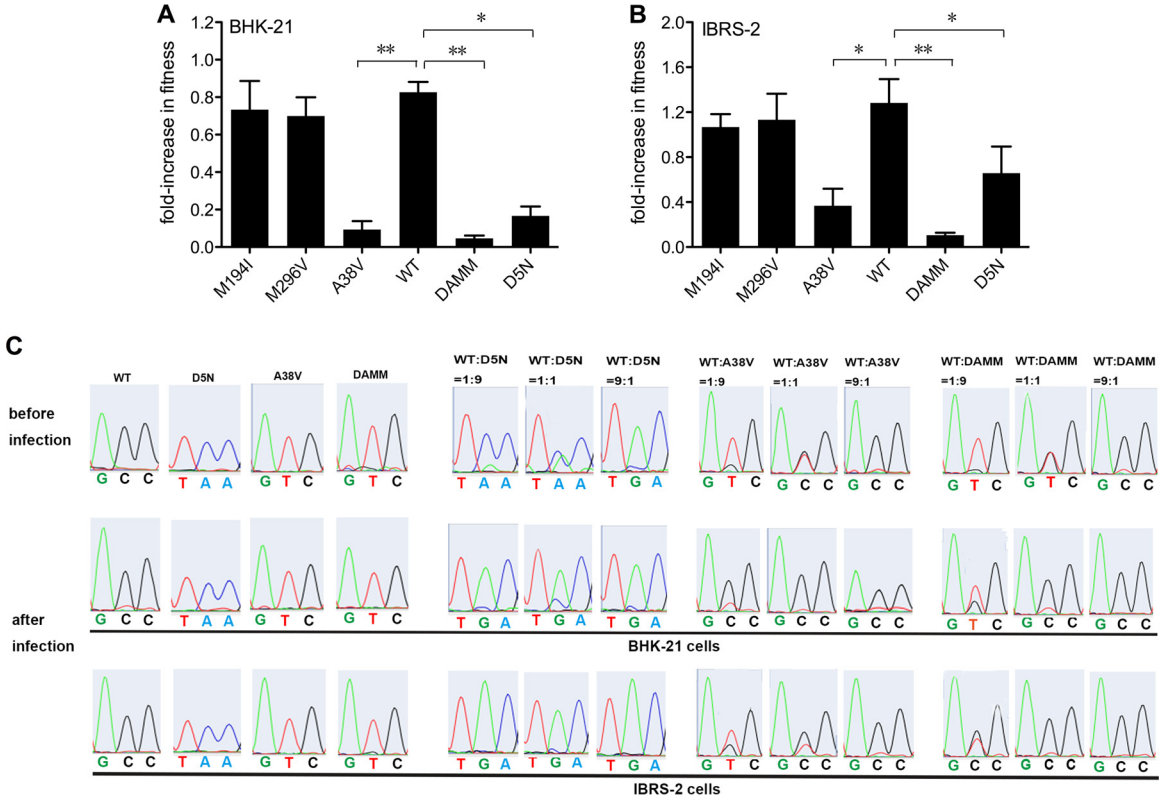

FIG 5 Competition assays comparing the relative fitnesses of each mutant and the WT virus. (A and B) Indirect assays. Each virus was mixed at a 1:1 ratio with the marked competitor, which contained the PstI restriction site, and inoculated in triplicate into BHK-21 (A) or IBRS-2 (B) cells at an MOI of 0.1. The progeny RNA was RT-PCR amplified, and restriction fragment length polymorphism assays were performed to determine the abundance of each competitor. Fitness is represented as the output-to-input ratio of the FMDV 3Dpol R84H variant to the marked competitor. A fitness value of <1 indicates that the mutant or the WT is less fit than the marked competitor. The mean values ± standard deviations are shown ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$ [determined by Student's t test]). (C) Direct assays. The D5N, A38V, or DAMM mutant was mixed with the WT at a ratio of 9:1, 1:1, or 1:9 and inoculated into BHK-21 or IBRS-2 cells at an MOI of 0.1 for 3 passages, after which the polymerase gene flanking position 5 or 38 was sequenced. The abundance of each competitor was measured as the height of the nucleotide encoding either the mutant (A or T nucleotide) or the WT (G or C nucleotide) in the sequencing chromatograms.

The DAMM mutant is significantly attenuated in suckling mice. We evaluated the effect of increased replication fidelity on the virulence of the D5N, A38V, and DAMM mutants in suckling mice. After inoculation with a dose of 0.01 $TCID_{50}$, no mice died in any of the mutant-inoculated groups or the WT FMDV-inoculated groups (Fig. 6A). After inoculation with a dose of 0.1 $TCID_{50}$, all mice died in the WT, M194I, and M296V virus-inoculated groups, but no mice died in the A38V and DAMM mutant-inoculated groups. One out of six mice died in the D5N mutant-inoculated group (Fig. 6B and C). After inoculation with a dose of 1 $TCID_{50}$, all mice died in the D5N virus-inoculated group, 4 out of 6 mice died in the A38V mutant-inoculated group, and no mice died in the DAMM mutant-inoculated group (Fig. 6D). At the highest dose (10 $TCID_{50}$), all mice in the DAMM mutant-inoculated group died (Fig. 6E). No mice died in the control group inoculated with PBS. Therefore, the DAMM mutant exhibited a 100-fold reduction in virulence compared with the WT virus, while the A38V and D5N mutants presented approximately 20-fold and 10-fold reductions in virulence, respectively.

More-attenuated variants have greater replication fidelity. The FMDV R84H variant, which has a 1.44-fold increase in RdRp fidelity, is not attenuated in suckling mice (31). Compared with WT FMDV, the newly isolated RdRp D5N and A38V variants and the constructed DAMM variant showed 1.51-, 1.65-, and 1.88-fold enhancements in replication fidelity, respectively, and exhibited 10-, 20-, and 100-fold viral attenuation in suckling mice, respectively (Fig. 7). Together, these data demonstrate that the extent of attenuation of virulence correlates positively with replication fidelity.

The DAMM mutant exhibits replication defects in bovine testis cells. The studies described above demonstrated that the high-fidelity D5N, A38V, and DAMM mutants had extremely decreased fitness in BHK-21 and IBRS-2 cells and were attenuated in suckling mice. We next used primary bovine testis cells, primary bovine kidney cells, and bovine thymus cells to examine whether restricted genetic diversity influenced the replication of FMDV mutants in their permissive host cells (Fig. 8A to C). The D5N, A38V, M194I, and M296V mutants showed viral titers similar to those of the WT in all cell types. Compared with the WT titer in primary bovine testis cells (6.75 $TCID_{50}$/ml), the DAMM mutant titer was decreased by 100-fold (4.75 $TCID_{50}$/ml; $P = 0.0035$ by Student's t test). No significant differences in viral titers were observed for the DAMM mutant in primary bovine kidney cells and bovine thymus cells. Thus, the reduction in DAMM mutant infectious virus progeny occurred only in primary bovine testis cells.

To further examine whether the significantly lowered viral titer of the DAMM mutant was associated with impaired viral RNA synthesis, primary bovine testis cells were inoculated with the WT

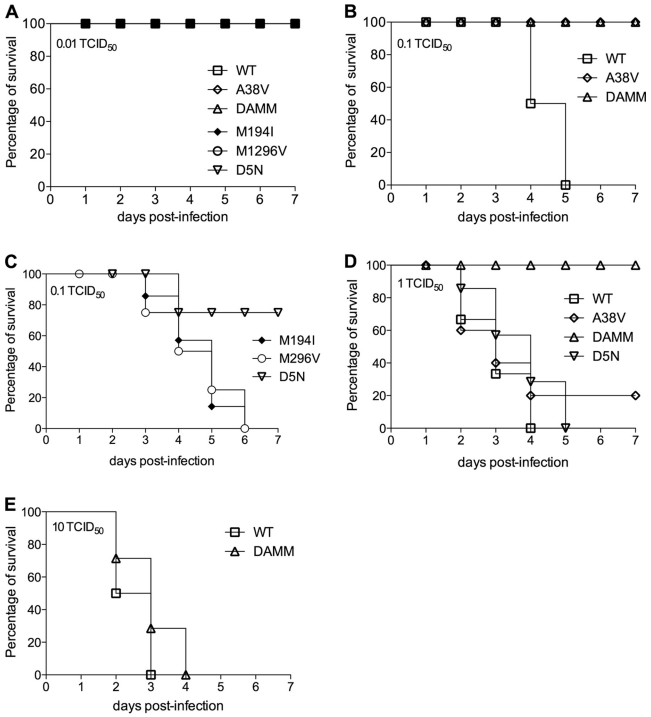

FIG 6 Virulence of rescued mutants in suckling mice. A total of 19 groups of 3-day-old BALB/c suckling mice were inoculated cervicodorsally with 100 μl of each mutant or WT FMDV diluted to 0.01 $TCID_{50}$ (A), 0.1 $TCID_{50}$ (B and C), 1 $TCID_{50}$ (D), or 10 $TCID_{50}$ (E). Animal deaths were scored for up to 7 days after inoculation, and survivors were euthanized ($n = 6$ to 9 for each group).

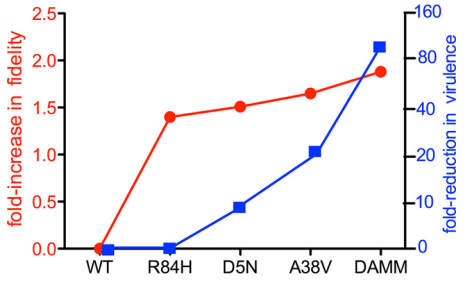

FIG 7 Correlation between RdRp fidelity and viral virulence. Each rescued variant exhibited different increases in replication fidelity (red line) and different reductions in virulence compared to the WT virus (blue line).

or mutant viruses in parallel, and the cell cultures were harvested at different time points for real-time PCR amplification. RNA synthesis by the DAMM mutant was significantly reduced compared to that of WT FMDV ($P = 0.0032$ by repeated-measures ANOVA) (Fig. 8D), while RNA synthesis by the other 4 mutants was unchanged (Fig. 8E). These results indicate that the DAMM mutant exhibits a growth defect in bovine testis cells.

DISCUSSION

Attenuated RNA virus variants can be obtained by modulating the replication fidelity of RdRp. These variants are ideal tools to examine viral evolutionary dynamics. Studies of high-fidelity RdRp mutants of PV, CVB3, CHIKV, and FMDV have demonstrated that (i) increased polymerase fidelity results in viral tolerance to mutagens, (ii) different yet broad-spectrum mutagen resistance occurs, and (iii) not all mutants are attenuated in animal models (23, 24, 27, 28, 31). In this study, attenuated FMDV high-fidelity RdRp variants showing different degrees of polymerase fidelity were isolated and examined in the context of polymerase fidelity and mutagen resistance. The inability of the D5N, A38V, and DAMM mutants to tolerate the 5-FU and AZC mutagens suggested that the high-fidelity variants did not have a broad range of mutagen resistance. As demonstrated by the A38V and DAMM mutants, higher replication fidelity was associated with stronger resistance to ribavirin. Based on the quantitative analysis of polymerase fidelity and virus virulence, our results confirm that virulence attenuation in mutagen-resistant mutants is proportional to the enhancement of replication fidelity.

Genetic variability in RNA viruses is linked to alterations in viral pathogenesis (27). Specifically, the expanded quasispecies diversity of a mutagen-induced $G64S^{eQS}$ population (i.e., a G64S population with expanded quasispecies) results in higher neurotropism and pathogenesis than a G64S population with the restricted quasispecies. Vignuzzi et al. first proposed the quasispecies theory, which proposes that the diversity of a viral quasispecies determines its pathogenesis through cooperative interactions in a viral population (29). In our previous (31) and present studies on mutagen selection, the 4 FMDV polymerase mutants showed 1.44- to 1.88-fold-enhanced replication fidelity, resulting in 10- to 100-fold-reduced virulence. Importantly, a positive correlation between replication fidelity and attenuation was observed, as briefly depicted in the schematic diagram based on the general model (40) (Fig. 9). We have proposed a rational hypothesis that viral virulence can be attenuated only by increasing polymerase fidelity, to the point where restricting quasispecies diversity affects the ability to generate the appropriate adaptive or escape mutations within a specific RNA virus population. In this paper, we confirmed that quasispecies diversity determines virus virulence via the isolation of several independent FMDV high-fidelity variants in response to mutagen exposure. Although selective pressure undoubtedly plays a key role in altering quasispecies diversity in RNA virus populations, a role of the complex biological processes that occur in both the viral pathogen and the host cannot be ruled out. Thus, the detailed mechanisms of replication fidelity and its effect on virulence remain to be explored.

Our current study demonstrated that the FMDV polymerase mutation impairs viral virulence in suckling mice. While cattle are undoubtedly the most ideal model for assessing FMDV virulence, a biosafety level 3 (BSL3) laboratory is required to handle large animals, which is not available at our institute. For the primary evaluation of FMDV virulence, the suckling mouse model is economical and widely accepted (37, 44, 45). The attenuation of virulence in a mouse model encourages further testing in the FMDV natural host.

RNA viruses have high error rates during virus replication, and the resulting quasispecies could contribute to the survival of the virus population in the presence of selective pressure. Although the fitness of the CVB3 high-fidelity A372V mutant has not been documented, most high-fidelity RdRp variants have decreased *in vitro* fitness (23, 24, 27, 46). In accordance with the quasispecies theory (40), the D5N, A38V, and DAMM mutants, which had unchanged replication kinetics but genetically less diverse populations, occupy lower and narrower peaks in the fitness landscape.

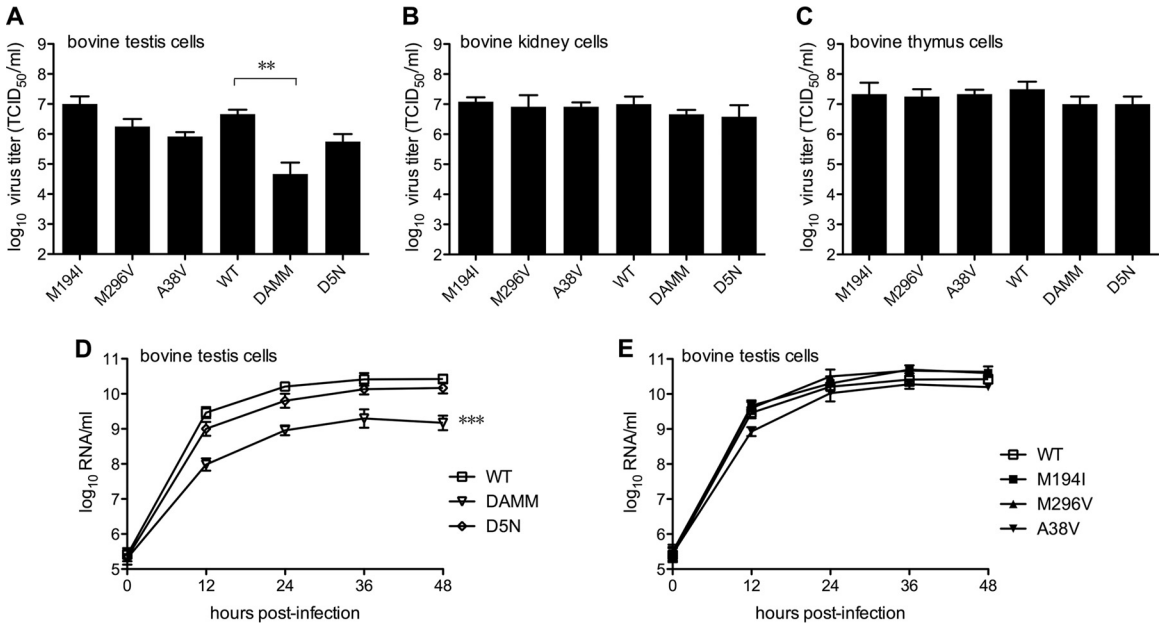

FIG 8 Viral titer and RNA synthesis for each mutant or the WT. (A to C) Viral titer. Primary bovine cells, bovine kidney cells, or bovine thymus cells in 96-well plates were continuously inoculated with diluted mutant viruses or WT FMDV at 50 μl/well. After CPEs were observed, the viral titer was determined as the $TCID_{50}$ per ml. The mean values ± standard deviations are shown ($n = 3$) (**, $P < 0.01$ by Student's t test). (D and E) RNA synthesis. Primary bovine testis cells, primary bovine kidney cells, or bovine thymus cells in 12-well plates were incubated with each mutant or WT FMDV at an MOI of 0.1 for 1 h, washed 3 times, and supplemented with DMEM. The infected cell cultures were harvested at specific time points. Cellular RNA was extracted, and real-time PCR amplification was performed. The mean values ± standard deviations are shown ($n = 3$) (***, $P < 0.001$ by repeated-measures ANOVA).

Specifically, we determined that higher polymerase fidelity was associated with lower fitness. Taken together, these observations demonstrate that restricting the genetic diversity of an RNA virus by increasing its fidelity compromises the ability of the virus to adapt to its changing environment and that, conversely, naturally high mutation rates confer an evolutionary advantage to RNA viruses. These findings are consistent with the idea that RNA viruses have evolved suboptimal viral polymerase fidelity to permit rapid evolution and adaptability (40).

Single-amino-acid substitutions at position 64/359, 372, 483, or 84 alter the fidelity of the RdRp of PV, CVB3, CHIKV, or FMDV, respectively (23, 24, 26, 28, 31). The residue at position 5 in the polymerase protein is critical for the stability of the elongation complex in PV and CVB3 (47, 48). Our results demonstrate that residue 5 in the polymerase protein of FMDV is involved in controlling RdRp fidelity without compromising viral replication (Fig. 2 and 3). These observations suggest that RdRp residue 5 in picornaviruses has multiple effects on viral biology. In addition, the DAMM mutant exhibited higher fidelity than the D5N and A38V mutants, suggesting a type of synergy for polymerase fidelity enhancement. Although the aim of this study was to isolate FMDV high-fidelity variants and explore the correlation between quasispecies diversity and virulence, it is unclear if the increase in DAMM fidelity is due only to the D5N and A38V amino acid substitutions or if there is a synergistic contribution from the M194I and M296V substitutions.

An RNA virus with higher tolerance to mutagens could result from increased replication fidelity (23, 24, 26), specific discrimination against the mutagen (25), or increased mutational robustness (17). Increased polymerase fidelity in the mutagen-isolated RdRp variants is usually hypothesized to be responsible for their resistance to mutagen treatments (33). Specifically, the ribavirin-isolated PV G64S, ribavirin- or AZC-isolated CVB3 A372V, and 5-FU-isolated FMDV R84H variants resist all 3 types of mutagen, AZC, and 5-FU (24, 26, 31). In contrast, the ribavirin- or 5-FU-isolated CHIKV C483Y variant is resistant to ribavirin and 5-FU (23); it is unknown if this variant is also resistant to broad-spectrum mutagens. However, not all high-fidelity variants have been completely evaluated for broad-spectrum mutagen resistance, and there are discrepancies in the reported resistance of these variants. In this study, the several FMDV high-fidelity variants that we isolated with ribavirin exhibited restricted broad-spectrum mutagen resistance compared with the R84H variant. All these results suggest that restricted resistance to mutagens for high-fidelity variants is the exception, while broad resistance is the standard, as has been demonstrated by the ribavirin-isolated FMDV M296I variant showing restricted resistance (49). It was believed that the ability of the isolated RdRp variants to identify natural or unnatural nucleotides was associated with the resulting structural changes of the mutant polymerases. For instance, amino acid 64 in the PV polymerase, which is remote from the active site, modulates polymerase fidelity via a conformational change in the polymerase structure (50). Thus, it is tempting to hypothesize that the differences in mutagen resistance of the isolated high-fidelity RdRp variants may be attributable, at least in part, to the structural characteristics of the mutagen that was used to select the variants.

In previous studies, all the high-fidelity RdRp variants generated through single-amino-acid substitutions did not exhibit replication defects in virus-infected cells (23, 24, 27, 31). Our current study, which constructed some high-fidelity RdRp variants generated through single- and combined-amino-acid substitutions, revealed that although the RdRp mutants produced similar infec-

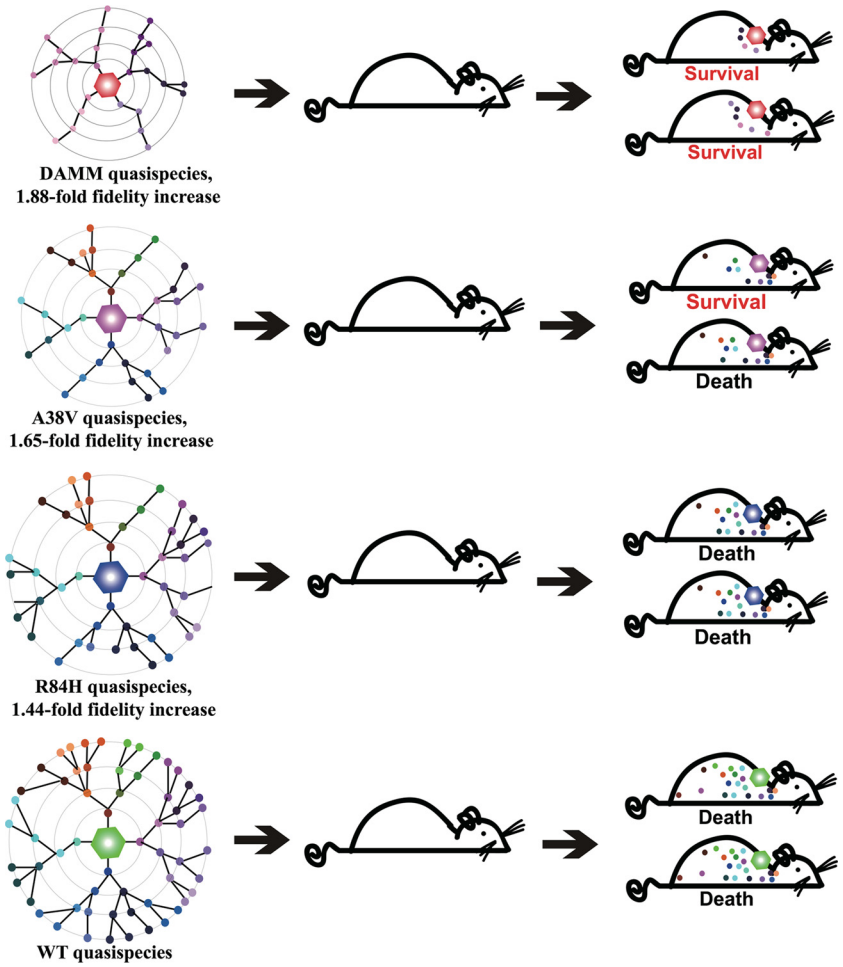

FIG 9 Schematic diagram of viral quasispecies diversity affecting viral virulence. Suckling mice were infected with WT virus or the R84H, A38V, or DAMM mutant virus (from bottom to top). Compared with the WT virus, the mutants had different degrees of enhanced replication fidelity, resulting in different restrictions in genetic diversity. Although all mice received the same viral titers of FMDV, only those infected with diverse quasispecies, such as the WT or R84H populations, developed the disease. Subpopulations within the diverse quasispecies cooperated with each other to facilitate their transmission in mice.

tion titers in bovine kidney cells and bovine thymus cells, the DAMM mutant was significantly restricted with regard to infection and RNA synthesis in primary bovine testis cells. These results suggest that the combination of the 4 mutations resulted in the replication defect of the DAMM mutant.

In this study, we explored the modulation of FMDV polymerase fidelity and its correlations with fitness and virulence. The results provide insight into the role of replication fidelity in the evolutionary dynamics of RNA viruses. Knowledge of the replication fidelity phenotypes of the mutants may be useful for the preparation of candidate live, attenuated FMDV vaccines, as the enhanced replication fidelity due to these mutations may help to improve the stability and safety of live, attenuated FMDV vaccines. However, it is still unclear what degree of RdRp fidelity is required for greater attenuation, how to turn the most attenuated mutant into a vaccine vector, and which mutagen is best for selecting high-fidelity RdRp mutants for each specific virus.

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