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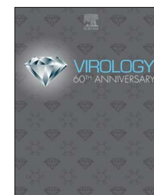

Foot-and-mouth disease virus type O specific mutations determine RNA-dependent RNA polymerase fidelity and virus attenuation

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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.

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 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 picornaviruses, 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

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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.

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