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An increased replication fidelity mutant of foot-and-mouth disease virus retains fitness *in vitro* and virulence *in vivo*

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ABSTRACT

In a screen for RNA mutagen-resistant foot-and-mouth disease virus (FMDV) strains, we isolated an FMDV mutant with RNA-dependent RNA polymerase (RdRp) R84H substitution. This mutant, selected under the mutagenic pressure of 5-fluorouracil (5-FU), is resistant not only to 5-FU but also to other two RNA mutagens, 5-azacytidine and ribavirin, suggesting that the RdRp R84H mutant is a high fidelity variant. Subsequently, the increased fidelity of this mutant was verified through analysis of mutation frequency, which revealed a 1.4-fold enhancement in RdRp fidelity compared with the wild-type virus. Further studies indicated that the R84H mutant exhibited slightly increased fitness *in vitro*, and its virulence was not reduced in suckling mice. These results indicated that an increase in RdRp fidelity does not always correlate with reduced virus fitness and virus attenuation. Thus, this isolated R84H mutant provides a new platform to examine the evolutionary dynamics of fidelity-changing RNA viruses, such as mutagen resistance, fitness and virulence.

1. Introduction

Nucleoside analogue mutagens, such as 5-fluorouracil (5-FU), 5-azacytidine (AZC) and ribavirin, are misincorporated into viral genomes during RNA synthesis, resulting in a significant increase in the frequency of deleterious mutations due to mispairing in subsequent replication cycles (Airaksinen et al., 2003; Chen et al., 2004; Crotty et al., 2001; Ruiz-Jarabo et al., 2003; Schaaper, 1998). 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 error or lethal mutations (Crotty et al., 2002; Goris et al., 2007; Graci et al., 2012; Loeb and Mullins, 2000; Pariente et al., 2005). The resulting lethal mutations have been exploited to develop efficacious antiviral strategies that rely on viral replication errors (Anderson et al., 2004; Crotty et al., 2001), as demonstrated with poliovirus (PV) and other RNA viruses (Chung et al., 2007; Loeb and Mullins, 2000; Martin et al., 2008; Sierra et al., 2000).

Interestingly, mutagens delivered at appropriate concentrations that do not give rise to large-scale lethal mutagenesis in the viral genome can be used to select mutagen-resistant variants of RNA-dependent RNA polymerase (RdRp) in RNA viruses (Coffey et al., 2011; Levi et al., 2010; Pfeiffer and Kirkegaard, 2003; Sierra et al., 2007). Using this strategy, three mutagen-resistant mutants with increased RdRp fidelity, including G64S of PV (Pfeiffer and Kirkegaard, 2003), A372V of coxsackie virus B3 (CVB3) (Levi et al., 2010) and C483Y of the Chikungunya virus (CHIKV) (Coffey et al., 2011), have been isolated. Although virulence of the CVB3 A372V mutant has not been reported, attenuation of the PV G64S mutant and the CHIKV C483Y mutant was documented (Coffey et al., 2011; Vignuzzi et al., 2008).

To date, despite some attempts (Arias et al., 2008; Sierra et al., 2007), high fidelity 3D polymerase (3Dpol) variants of FMDV have not been successfully selected using RNA mutagens. In this study, we isolated a high fidelity 3Dpol R84H mutant with increased resistance to 5-FU, AZC and ribavirin. The phenotype of this mutant such as viral fitness *in vitro* and virulence *in vivo* are also discussed.

2. Materials and methods

2.1. Cells and viruses

BHK-21 (baby hamster kidney cell line) and IBRS-2 (swine kidney cell line) cells were grown in Dulbecco Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL) at 37 °C in 5% CO_2. Asia1/YS/CHA/05, the wild-type (WT) strain of serotype Asia1 FMDV, was generated from the infectious cDNA clone pAsi (Wang et al., 2011).

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2.2. Virus passage under mutagen selection pressure

Isolation of RdRp variants by passages of FMDV under mutagen selection pressure was performed as previously described (Beaucourt et al., 2011). Briefly, BHK-21 cells were pretreated with 800 μM 5-FU (Sigma) for 3 h, incubated with FMDV for 1 h at an MOI of 0.05 and subsequently treated with 800 μM 5-FU again. FMDV was harvested within 72 h post-infection (PI), and the progeny viruses were subjected to the next round of treatment for a total of 32 passages. The harvested virus stocks were sequenced at intervals of five passages and from the 32nd passage.

2.3. Construction of a recombinant 3Dpol plasmid with the R84H substitution

The pAsi plasmid was digested with MluI and EcoRV, which was ligated into a pOK12 using T4 DNA ligase (New England BioLabs). The resulting plasmid containing the 3Dpol gene was used for site-directed mutagenesis with primers 5′-GCCGACTACGCGTCGCACTTGCACAGCGTGCTG-3′ and 5′-CAGCACGCTGTGCAAGTGCGACGCGTAGTCGGC-3′. The underlined base in each primer corresponds to the mutated nucleotide. The positive plasmid bearing the R84H substitution in 3Dpol was digested with MluI and EcoRV and reintroduced into the pAsi. The resulting recombinant plasmid was used for *in vitro* transcription and transfection.

2.4. In vitro transcription and transfection

The plasmid was linearized by digestion with EcoRV, and transcripts were generated using the RiboMAX™ Large Scale RNA Production Systems-T7 kit (Promega). After transcription, the reaction mixtures were each treated with 1 U RQI DNase/μg RNA (Promega). BHK-21 cells were transfected with 5 μg of *in vitro*-transcribed RNA using the Effectene® Transfection Reagent (Qiagen). After 48-h incubation at 37 °C, viruses were harvested via three freeze–thaw cycles.

2.5. RNA mutagen assays

BHK-21 cell monolayers were pretreated with 1100 μM AZC (Sigma) and 50 μM ribavirin (Sigma) for 3 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 R84H variant at an MOI of 0.01 for 1 h and were subsequently treated with the same mutagen concentration as during the pretreatment. Within 72 h PI, the infectious titer was determined using the $TCID_{50}$ assay.

2.6. Replication kinetics and RNA synthesis of FMDV WT versus the R84H mutant

One-step growth curves were performed in both BHK-21 and IBRS-2 cells as described (Agudo et al., 2010). Mean titers and standard deviations were calculated from three independent experiments. For FMDV genome copy measures, quantification of FMDV genomic copies was conducted by a previously described real-time RT-PCR assay (Goris et al., 2009; Shaw et al., 2007). Both standard curves ($y = -3.77x + 42.02$; $R^2 = 0.96$) in BHK-21 cells and ($y = -4.36x + 48.96$; $R^2 = 0.97$) in IBRS-2 cells were generated using three replicates of *in vitro* transcribed genomic RNA.

2.7. Direct and indirect competition fitness assays

Direct competition fitness assay was carried out as previously described (Govorkova et al., 2010), the rescued FMDV R84H mutant was mixed with its parental virus Asia1/YS/CHA/05 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 three passages. Viral RNA was extracted, and a region flanking 3Dpol amino acid 84 was amplified by RT-PCR for sequencing. The abundance of each competitor was measured as the height of the nucleotide encoding either the FMDV WT (nucleotide G) or the R84H mutant (nucleotide A) in sequencing chromatograms.

Based on previous methodologies (Coffey and Vignuzzi, 2011) with minor modification, Indirect competition fitness assay was performed with or without pressure of monoclonal antibody 3E11 (Wang et al., 2011). In brief, the fitness of rescued FMDV R84H mutant was indirectly compared with that for WT FMDV by competing each variant in a 1:1 ratio against the same marked competitor, a WT FMDV variant with a PstI restriction site at 249 amino acid position of 3Dpol. RNA was extracted from BHK-21 or IBRS-2 cells culture supernatants twelve hours PI at an MOI of 0.1, and a restriction fragment length polymorphism assay was performed. Fitness is represented as output:input ratio of the 3Dpol R84H mutant to the marked competitor FMDV.

2.8. Measurement of FMDV mutation frequency by sequencing

FMDV RNA was extracted and cDNA was generated by reverse transcription of total RNA using PrimeScript® Reverse Transcriptase (Takara). 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) using the following primers: 5′-ACGTTGAGGTGACCGCTGTTG-3′ (upstream) and 5′-AGTCGGGTCCGTGTTTTGTTG-3′ (downstream). The PCR product was purified and cloned into the pMD18-T vector (Takara) for sequencing. The number of mutations per 10^4 nucleotides sequenced was determined according to the total number of mutations identified in each population over the product of the total number of nucleotides sequenced for that population multiplied by 10^4. For each population, at least 55 partial P1 structural gene sequences of approximately 1800 nt per replicate were sequenced (Fig. 1A). Mutation frequencies (mutations per 10,000 nt) were determined as previously described (Beaucourt et al., 2011). For further sequencing, three replicates of the R84H mutant or WT FMDV were passaged five times in BHK-21 cells under the selection pressure of 1200 μM 5-FU. The mutation frequency of each sixth passage population under the pressure of 5-FU was determined using the same method as described above in the absence of 5-FU.

2.9. Analysis of rescued virus virulence in suckling mice

The animal experiments were approved by the Animal Ethics Committee of the Harbin Veterinary Research Institute, CAAS, China. BALB/c suckling mice were purchased from the Slac Laboratory Animal Co. Ltd, Shanghai, China. Three-day-old BALB/c suckling mice were divided into eight groups (10 mice per group) and were inoculated cervicodorsally with 100 μL of diluted virus from 1 $TCID_{50}$ to 0.001 $TCID_{50}$ in tenfold dilution series as previously described (Baranowski et al., 2003; Gutierrez-Rivas et al., 2008). The percentage survival of the animals was recorded every 12 h up to 8 days after inoculation.

3. Results

3.1. Generation of FMDV populations resistant to 5-FU

To select FMDV high fidelity variants, we passaged FMDV in the 5-FU-containing medium as described previously (Beaucourt et al., 2011) with minor modifications. Wild-type (WT) FMDV rescued from the infectious clone pAsi was serially passaged 32 times in

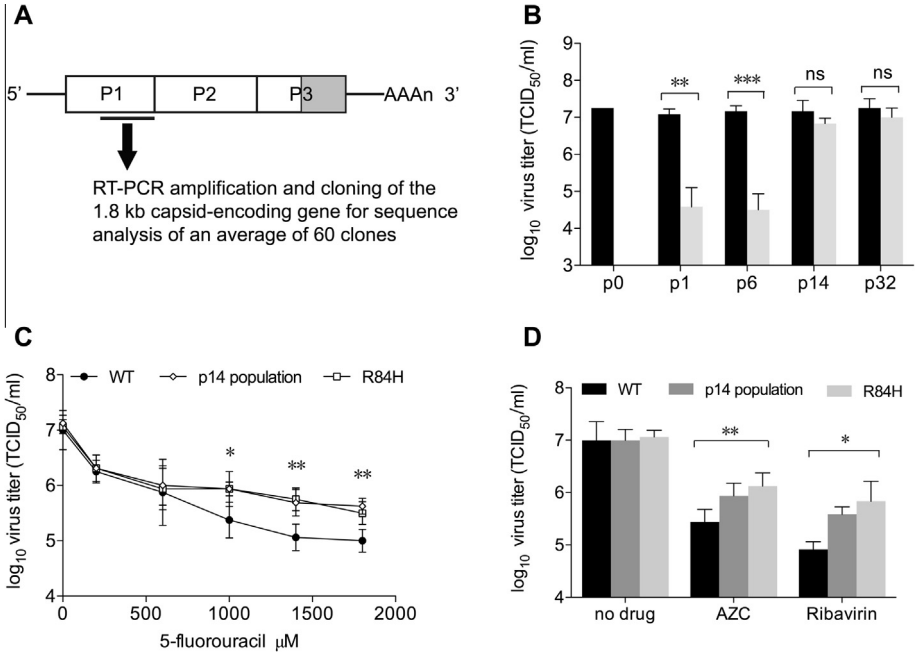

Fig. 1. Isolation of the RNA mutagen-resistant FMDV mutant. (A) Schematic of the FMDV RNA genome. The 3Dpol region is shaded gray. A 1.8-kb section of the viral capsid gene was RT-PCR-amplified and cloned for sequencing. Individual clones were used to determine the mutation frequencies presented throughout this work. (B) Generation of the RNA mutagen-resistant FMDV mutant. FMDV WT was serially passaged with (gray) or without (black) 800 μM 5-FU at an MOI of 0.01. p0 is the initial WT stock titer. Mean titers of harvests ± SD are shown (*t* test, $n = 4$, $^{**}P < 0.01$, $^{***}P < 0.001$). (C) Resistance of the FMDV R84H mutant to 5-FU. Matched titer of the R84H mutant, the p14 population or the FMDV WT was inoculated into quadruplicate wells with BHK-21 cells and increasing concentrations of 5-FU. Mean titers ± SD are shown (One-way ANOVA, $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$). (D) Resistance of the R84H mutant to AZC and ribavirin. BHK-21 cells pretreated with 1100 μM AZC or 50 μM ribavirin were infected with the R84H mutant, the p14 population or FMDV WT at an MOI of 0.01. A control infection (no drug) was also performed. Within 72 h after infection, progeny virus was titered by $TCID_{50}$ assay using BHK-21 cells. Mean virus titers ± SD are shown (One-way ANOVA, $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$).

the presence or absence of 800 μM 5-FU. After one passage (p1), the mean titer of the 5-FU-treated FMDV decreased by approximately 2.5 logs ($P < 0.01$) compared with the untreated FMDV (Fig. 1B). By passage 14 (p14), no significant reduction in titer was observed for the 5-FU-treated FMDV compared to the WT (Fig. 1B). Importantly, this phenotype was observed persistently from p14 to p32 (Fig. 1B), indicating that a variant resistant to 5-FU had been stably generated.

3.2. A single R84H amino acid substitution in 3Dpol confers FMDV resistance to 5-FU

To identify the mutation(s) conferring resistance to 5-FU, the 3Dpol gene was amplified from the 5-FU-selected 14th passage (the p14 population) and sequenced. The 3Dpol gene was chosen because polymerase point mutations are usually identified as mediators of mutagen resistance in RNA viruses (Arias et al., 2008; Coffey et al., 2011; Levi et al., 2010; Sierra et al., 2007; Vignuzzi et al., 2008). A single amino acid change from Arg to His at position 84 of the 3Dpol protein was observed from the 5-FU-selected p14–p32 population, suggesting that the 3Dpol R84H substitution is the principal mediator of the FMDV variant's resistance to 5-FU mutagenesis. FMDV R84H mutant was rescued to test the resistance to 5-FU over a range of concentrations. Compared to the FMDV WT, the R84H mutant exhibited significantly increased resistance to 5-FU treatment similar to the p14 population (Fig. 1C). The maximum difference in resistance to 1400 μM of 5-FU between the R84H mutant and the FMDV WT was 0.7logs (One-way ANOVA, $P = 0.0038$) within the range observed for high fidelity variants of picornaviruses (Levi et al., 2010). These results demonstrated that a single R84H amino acid substitution in 3Dpol mediates FMDV resistance to 5-FU.

3.3. The R84H substitution of 3Dpol also confers resistance to other RNA mutagens

Studies have indicated that if a polymerase mutagen-resistant variant is capable of resisting more than two nucleoside analogue mutagens, this variant may be regarded as a high fidelity mutant (Beaucourt et al., 2011). To test whether the R84H mutant was resistant to other RNA mutagens, the rescued R84H mutant was treated with two RNA mutagens: AZC (1100 μM) and ribavirin (50 μM). The FMDV WT and the p14 population were used as control. The results revealed that the R84H mutant similar to the p14 population was resistant to the two RNA mutagens tested (One-way ANOVA, $P = 0.0083$ and $P = 0.011$, respectively) (Fig. 1D), just as it was resistant to 5-FU, suggesting that the FMDV R84H mutant is a high fidelity polymerase variant.

3.4. The R84H mutant is a high fidelity variant of FMDV

To test whether the mutagen resistance of the R84H mutant was due to increased polymerase fidelity, the mutation frequencies of the R84H mutant and FMDV WT were compared using statistically analysis of Mann-Whitney test giving the more conservative*P* values (Levi et al., 2010). Mutations per 10,000 nt of the three replicates of the R84H mutant (2.42, 2.65 and 2.58) was significantly fewer than that of the FMDV WT (3.56, 3.88 and 3.59) in the absence of 5-FU (one-tailed Mann–Whitney test, $P < 0.05$) (Fig. 2A). Although the mutations per 10,000 nt of the R84H mutant increased to 9.90 after treatment with 1200 μM 5-FU, it still displayed significantly fewer mutations than that of the FMDV WT (12.53) (one-tailed Mann–Whitney test, $P < 0.05$) (Fig. 2B). These results further demonstrated that the R84H mutant is indeed a 3Dpol high fidelity variant.

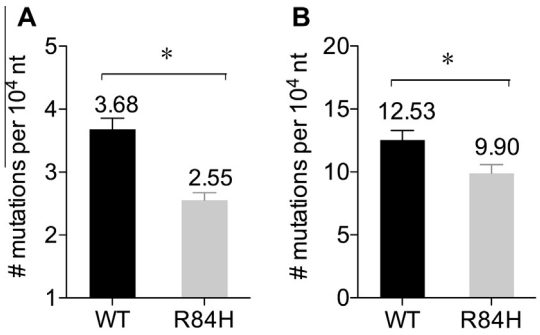

Fig. 2. Mutation frequencies of the R84H mutant and FMDV WT. Three independent stocks per virus were generated in 5-FU-free medium (A), and in medium containing 1200 μM 5-FU (B). A mean of sixty partial P1 sequences (approximately 100,000 nucleotides replicate^{-1}) were obtained. The mean mutation frequencies (number of polymorphisms per 10,000 nt sequenced) ± SD represent the averages of all the replicates; the same pattern of reduced mutation frequency for the R84H mutant (gray bar) compared to the WT (black bar) was observed for each replicate (one-tailed Mann–Whitney test, $n = 3$, $^*P < 0.05$).

In BHK-21 and IBRS-2 cells, no significant differences were observed in the production of infectious particles between the R84H mutant and FMDV WT (Fig. 3A and C). In addition, for viral RNA synthesis, the RNA produced from the R84H mutant infection was quantitatively similar to that of the FMDV WT in both cells (Fig. 3B and D). Thus, these results indicated that the reduced mutation frequency of the R84H mutant was not caused by a replication defect.

3.5. The R84H variant does not exhibit reduced fitnessin vitro

To assess whether the R84H substitution results in a fitness change for the R84H mutant*in vitro*, a comparison of fitness between the R84H mutant and FMDV WT was performed via two types of competition assays in both BHK-21 and IBRS-2 cells. In direct assays (Fig. 4A), after three successive passages, the 84H chromatogram peaks were slightly higher than the 84R peaks, indicating that the R84H variant had a slight fitness advantage over the FMDV WT in both types of cells. In addition, the fitness of the FMDV WT was neutral to the reference in BHK-21 cells via a more quantitative but indirect assay (Fig. 4B), whereas a slightly higher fitness of the R84H mutant than the FMDV WT was observed, consistent with results from direct competition assays. And the R84H mutant's fitness was higher than the FMDV WT that showed a slightly higher fitness than the reference in IBRS-2 cells. Further, under pressure of monoclonal antibody 3E11 with the dilution of 1:8192, the R84H mutant displayed similar competitive advantage to the WT virus compared the marked virus (Fig. 4B). Together these results indicated that the R84H mutant is slightly more fit than the FMDV WT or retains fitness in both types of cells.

3.6. The R84H fidelity variant does not exhibit attenuated virulence in suckling mice

To determine whether the R84H mutant would present reduced virulence, BALB/c suckling mice were inoculated with different $TCID_{50}$ as described previously (Baranowski et al., 2003; Gutierrez-Rivas et al., 2008). After inoculation with a dose of either 1 $TCID_{50}$ or 0.001 $TCID_{50}$, the survival percentages of the R84H mutant-inoculated group and the FMDV WT-inoculated group were identical (Fig. 5A and D). Although a discrepancy in the survival percentage was observed between the R84H mutant-inoculated group and the control group after inoculation with a dose of either 0.1 $TICD_{50}$ or 0.01 $TICD_{50}$ (Fig. 5B and C), no significant differences in virulence were observed between the two groups as evaluated with the Log-rank (Mantel–Cox) test. No mice died in the control group inoculated with PBS. These results revealed that no significant different virulence in BALB/c suckling mice was observed between the R84H mutant and FMDV WT.

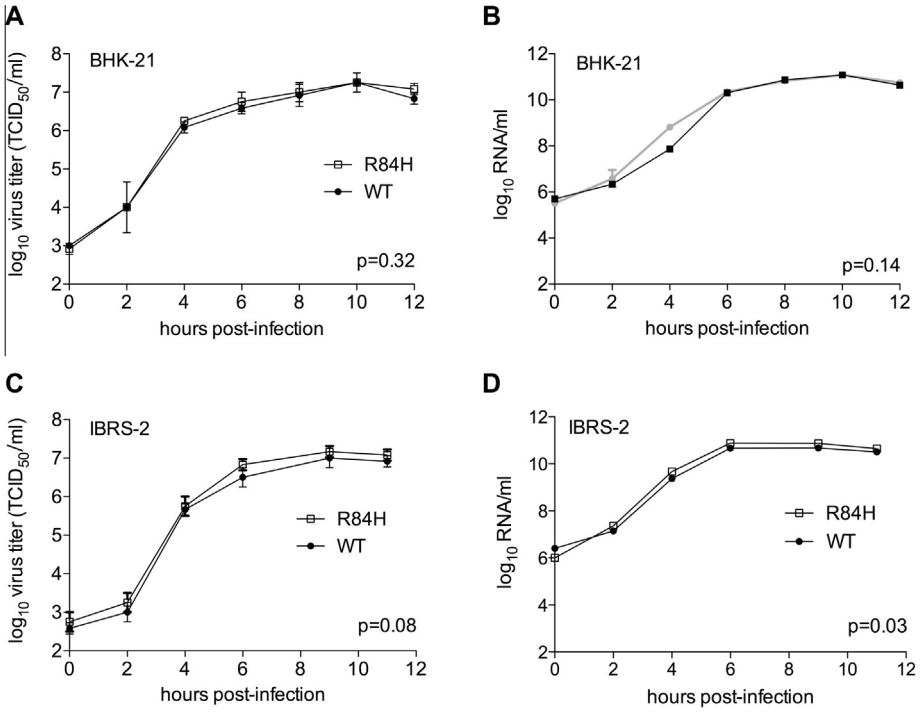

Fig. 3. One-step growth curves and RNA synthesis of the FMDV R84H mutant. BHK-21 and IBRS-2 cells were infected with the R84H mutant or FMDV WT at an MOI of 10. Virus harvested at different times was titered and expressed as a $TCID_{50}$ dose (A and C), and genome copy numbers were measured (B and D) for the same samples by real-time RT-PCR. Mean values ± SD and *P* values (repeated measures ANOVA, $n = 3$) are shown.

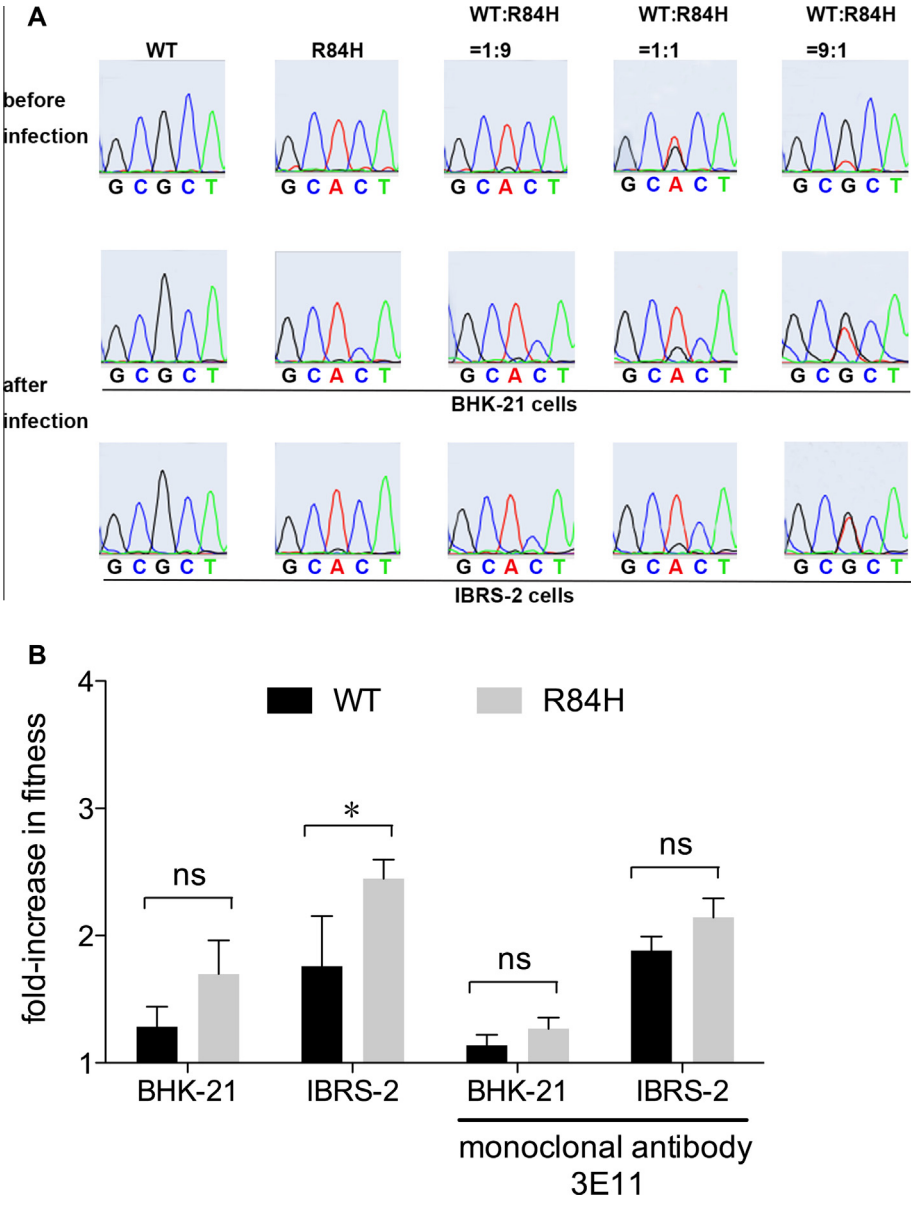

Fig. 4. Competition assays comparing the relative fitness levels of the R84H mutant and FMDV WT. (A) Direct assays. (B) Indirect assays. A fitness value > 1 indicates that the R84H mutant or the FMDV WT is more fit than marked competitor. Mean values ± SD are shown (*t* test, $n = 3$, $^{*}P < 0.05$).

4. Discussion

In this study, an FMDV 3Dpol R84H variant with resistance to three RNA mutagens was selected using 5-FU mutagenesis (Fig. 1C and D). Compared with the FMDV WT, this variant presented 20.9% and 30.7% lower mutation frequencies with (Fig. 2B) and without (Fig. 2A) 5-FU treatment, respectively. Moreover, the R84H mutant's *in vitro* fitness and virulence *in vivo* were not significantly impacted by the decreased genetic heterogeneity of the population.

The intrinsically low fidelity of viral RdRps generates a large number of spontaneous genomic mutations, the majority of which are deleterious to viral replication capacity (Lauring and Agudo, 2010). For instance, approximately 60% of spontaneous mutations in vesicular stomatitis virus (VSV) are deleterious (Sanjuan et al., 2004). Thus, the high fidelity R84H mutant would produce fewer spontaneous deleterious mutations, thereby providing a slight increase in fitness. However, previous studies have demonstrated that high fidelity RdRp variants are less fit in new or more complex environments due to the generation of fewer adaptive mutations derived from restricted genetic diversity (Coffey et al., 2011; Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006, 2008; Weeks et al., 2012). These contrary results suggest that, in addition to RdRp fidelity and its impact on adaptive and deleterious mutations, other factors, including single-nucleotide substitutions (Sanjuan et al., 2004) and mutational robustness (Lauring et al., 2012), responsible for virus fitness and virulence remain to be elucidated.

The rationally engineered PV RdRp mutant K359R, which exhibited higher fidelity than the G64S mutant, was more attenuated than the G64S mutant that was also re-evaluated to reveal similar virulence to wild-type PV (Weeks et al., 2012). For the FMDV R84H mutant, a 1.4-fold increase in fidelity might not have significantly attenuated the virus. Thus, we argue that the RdRp mutant exhibits an attenuated phenotype during *in vivo* infection only when its RdRp fidelity is increased to a relatively high level for specific RNA virus, although there are other factors that are involved in determination of virus virulence, such as mutational robustness (Lauring et al., 2012). Although use of suckling mice model for

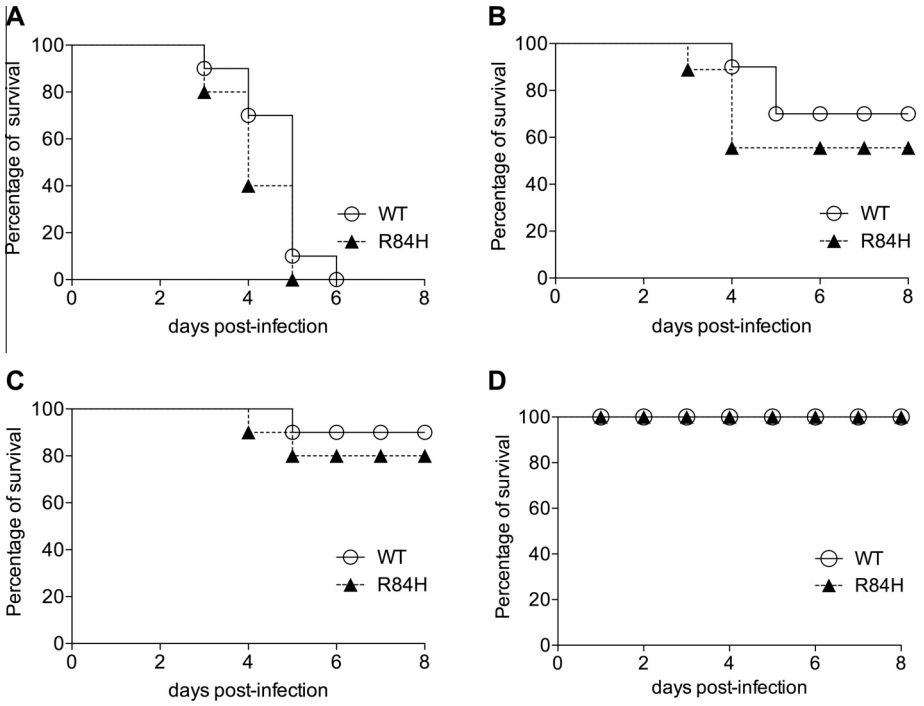

Fig. 5. No significant differences in virulence between the R84H mutant and FMDV WT were observed. Eight groups of 3-day-old BALB/c suckling mice were inoculated cervicodorsally with 100 μL of the R84H mutant or FMDV WT with 1 $TCID_{50}$ (A), 0.1 $TCID_{50}$ (B), 0.01$TCID_{50}$ (C) or 0.001 $TCID_{50}$ (D). Animal deaths were scored for up to 8 days after inoculation, and survivors were then euthanized (Log-rank (Mantel–Cox) test, $n = 10$ for each group, $P > 0.05$, no significant differences in virulence were identified between the R84H mutant and FMDV WT in all groups).

assessing FMDV virulence is acceptable (Garcia-Nunez et al., 2010; Gutierrez-Rivas et al., 2008; Rodriguez-Pulido et al., 2001), it is obviously that cattle is the best animal to evaluate the mutant's virulence.

In summary, we have selected a FMDV high fidelity 3Dpol variant, R84H, with slightly increased fitness but unaltered virulence. Further work is needed to explore the relationship between increased RdRp fidelity and evolutionary dynamics such as viral fitness and virulence.

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