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RESEARCH ARTICLE

# Effect of amino acid mutation at position 127 in 3A of a rabbit-attenuated foot-and-mouth disease virus serotype Asia1 on viral replication and infection

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**An amino acid mutation (R127→I) in the 3A non-structural protein of an FMDV serotype Asia1 rabbit-attenuated ZB strain was previously found after attenuation of the virus. To explore the effects of this mutation on viral replication and infection, the amino acid residue isoleucine (I) was changed to arginine (R) in the infectious cDNA clone of the rabbit-attenuated ZB strain by site-directed mutagenesis, and the R127-mutated virus was rescued. BHK monolayer cells and suckling mice were inoculated with the R127-mutated virus to test its growth property and pathogenicity, respectively. The effects of the R127 mutation on viral replication and virulence were analyzed. The data showed that there was a slight difference in plaque morphology between the R127-mutated and wild-type viruses. The growth rate of the mutated virus was lower in BHK-21 cells and its virulence in suckling mice was also attenuated. This study indicates that the R127 mutation in 3A may play an important role in FMDV replication *in vitro* and in pathogenicity in suckling mice.**

**KEYWORDS** **foot-and-mouth disease virus (FMDV); 3A protein; mutation; replication ability; virulence**

## INTRODUCTION

Foot-and-mouth disease (FMD) is a highly infectious disease of cloven-hoofed animals. The causative agent, foot-and-mouth disease virus (FMDV), is a member of the genus *Aphthovirus* within the family *Picornaviridae*, and can be divided into seven serotypes: O, A, C, Asia1, and Southern African Territories (SAT)1, SAT2, and SAT3 (Domingo E, et al., 2003; Grubman M J, et al., 2004). The FMDV genome includes a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (5′-UTR and 3′-UTR). Upon infection, the viral RNA is translated into a single polyprotein that is concurrently processed by three virus-encoded proteinases, leader ($L^{pro}$), 2A, and $3C^{pro}$, into the pre-cursors and consequent mature structural (VP1, VP2, VP3 and VP4) and non-structural ($L^{pro}$, 2A, 2B, 2C, 3A, 3B, $3C^{pro}$ and $3D^{pol}$) proteins (Belsham G J, 1993; Ryan M D, et al., 1989).

The FMDV serotype Asia1 has circulated widely in Asia since its detection for the first time in samples collected from India between 1951 and 1952 and from Pakistan in 1954 (Valarcher J F, et al., 2009). In China, the first outbreak of Asia1 FMD was recorded in Yunnan province in 1958 (Du J, et al., 2007; Xin A, et al., 2009), and the viral isolate was named the ZB (Zhongguo Baoshan) strain. Subsequently, the wild-type virulent ZB strain was inoculated into suckling rabbits for more than 100 passages, and the rabbit-passaged virus was shown to be attenuated in cattle. A live rabbit-attenuated ZB

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vaccine was produced to prevent FMD serotype Asia1 outbreaks on the border between China and Myanmar in the 1960s to 1990s, and showed perfect efficacy, confirming the safety of the live attenuated ZB vaccine. However, owing to the defects of the attenuated vaccine, such as the possibility of contamination and the virulent recovery of vaccine virus, this live attenuated vaccine was discontinued in the early 1990s.

The non-structural protein 3A is a conserved protein of 153 amino acids (aa) in most FMDVs examined to date (Carrillo C, et al., 2005). It was previously demonstrated that the Q44→R mutation in 3A of FMDV strain C-S8cl can mediate adaptation of FMDV to guinea pig (Nunez J I, et al., 2001). The 3A protein has been shown to be associated with egg-adapted derivatives of attenuated FMDV from cattle (Giraudo A T, et al., 1990), and deletions in 3A have been found to be associated with FMDV attenuation in cattle and high virulence in pigs (Beard C W, et al., 2000; Pacheco J M, et al., 2003). The complete sequence of 3A in As1/CHA/05 is an indispensible component for its replication in calf kidney cells; virus with a 10 aa deletion failed to replicate in primary calf kidney cells *in vitro* (Li S, et al., 2010). It was recently shown that a partial deletion in 3A could attenuate FMDV in cattle (Pacheco J M, et al., 2013) and a cellular protein, DCTN3, which is a specific bind-ing partner for FMDV 3A protein, was shown to be important for viral virulence in cattle (Gladue D P, et al., 2013).

To understand the virulence determinants of the attenuated ZB strain, we previously identified genomic changes between the attenuated FMDV ZB strain and its parental virulent strain (Xin A, et al.,2014). Comparison of those genomic sequences indicated that no deletion or insertion in the 3A protein occurred during the attenuation process, whereas five common amino acid mutations (E78→G, H80→C, K84→N, A97→P, and R127→I) were observed after attenuation. These amino acid substitutions resulted in a less charged and more hydrophobic 3A protein (Xin A, et al., 2014).

To investigate the effects of the R127 mutation in 3A on viral replication and infection, we developed a mutated virus (rZB-3A-R127I), in which the residue isoleucine (I) at position 127 of 3A protein was changed to arginine (R), using a full-length infectious cDNA clone derived from the rabbit-attenuated ZB/CHA/58/(att) strain (Xin A, et al., 2009), and evaluated its replication ability in cell culture and its virulence in suckling mice compared with its parental virus (rZBatt). Our results showed that the R127 mutation in the 3A protein of the attenuated ZB strain affected its *in vitro* replication ability, and also resulted in different virulence activities between the R127-mutated virus and its wildtype virus in suckling mice. This report presents direct evidence showing that the R127 mutation is related to the virulence change in suckling mice, which might be one of the key determinants of ZB strain attenuation. Special consideration should be given to the 3A protein in viral virulence studies.

## MATERIALS AND METHODS

### Plasmids and cells

The plasmid DNA for ZB/CHA/58(att) (pZBC12) has been described previously (Xin A, et al., 2009), and was modified. Briefly, the ZB/CHA/58(att) full-length genome was assembled between the *Cla* I and *Eco* RV restriction sites of the modified pOK12 vector backbone. An additional restriction site was generated in the genome by mutating the nucleotide T to A at position 6907 to produce the *Pst* I restriction site as the genetic marker of the recovered virus. The modified plasmid was named pZBatt. Viruses were propagated in BHK-21 cells, which were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 5% $CO_2$.

### Laboratory animals

BALB/c mice (Laboratory Animal Research Center of Kunming Medical University) at 3 days old were used for animal trials in this study. All the animal trials were approved by the Yunnan Laboratory Animal Administration Authority.

### Construction of mutation plasmids

The plasmid pZBatt was used as template for site-directed mutagenesis according to the Fast Mutagenesis System (Beijing TransGen Biotech. Co., Ltd, China). The primers 3A-R127-F (GTTTCAGAGAGAGAACTCTCCCG, forward primer) and 3A-R127-R (CTCTCTCTGAAACCAACAGTGCT, reverse primer) were used to introduce the specific mutation. For PCR, the 50 μL reaction mixtures comprised 10 μL 5 × *FastPfu* buffer, 1 μL 10 mmol/L dNTP, 2 μL 3A-R127-F, 2 μL 3A-R127-R, 1 μL *FastPfu* DNA polymerase, 33.5 μL sterile double-distilled $H_2O$, and 0.5 μL pZBatt. The PCR protocol used was: 5 min of pre-denaturation at 95 °C, 20 cycles of 95 °C for 30 sec, annealing 55 °C for 30 sec, and extension 72 °C for 6 min, followed by a final extension at 72 °C for 10 min. The PCR products were digested with 1 μL DMT Enzyme at 37°C for 1 h. After digestion, the mutated plasmid was transformed into *Escherichia coli* DMT competent cells and grown at 37°C in the presence of kanamycin. The constructed mutation plasmid, designated pZB-3A-R127I, was verified by DNA sequencing.

### Rescued viruses from cDNA clones

The mutation-containing plasmid pZB-3A-R127I and wild-type plasmid pZBatt were linearized with the

restriction endonuclease *Eco* RV, and used as templates for RNA synthesis with the RiboMAX Large Scale RNA Production Systems-T7 Kit (Promega, USA) in accordance with the manufacturer's instructions. BHK-21 cells were transfected with 5 μg of *in vitro* transcribed RNA using X-tremeGENE HP Transfection reagent (Roche, Germany). The cells were incubated for 4 h at 37°C and then the medium was replaced with DMEM plus 2% FCS. After 72 h, culture medium was harvested by three cycles of freeze–thawing, and passaged three more times in fresh BHK-21 cells. Rescued viruses were named rZB-3A-R127I and rZBatt, respectively.

## Sequencing of the R127-mutated virus

BHK-21 cells infected with the rZB-3A-R127I were treated with RNAiso Plus (TaKaRa Biotech. Co., Ltd) and the extracted total RNA was reverse-transcribed with TransScript II One-Step gDNA Removal and cDNA Synthesis Supermix (Beijing TransGen Biotech Co., Ltd. China) using the specific primers as described previously (Xin A, et al., 2009). Fragments covering the full-length genomic of rZB-3A-R127I were PCR-amplified, and the amplified products were subjected to nucleotide sequencing to confirm the introduction of the amino acid substitutions and the absence of any other changes.

## Immunofluorescence analysis

The rescued viruses were detected by an immunofluorescence assay (IFA) using monoclonal antibody (mAb) 3E11 (kindly provided by Prof. Yu Li, Harbin Veterinary Research Institute, CAAS) as described previously (Xue M, et al., 2012). BHK-21 cells were infected with rZB-3A-R127I or rZBatt at a multiplicity of infection (MOI) of 0.1 in 96-well plates. Eight hours post-infection (PI), cell monolayers were fixed with ice-cold anhydrous ethanol for 10 min at −20°C and air-dried, then 50 μL of mAb 3E11 (1:200 dilution in PBS pH7.2) was added to each well and incubated for 1 h at 37°C. The monolayers were washed with PBS with 0.1% (v/v) Tween 20, followed by two washes with PBS alone, then incubated with FITC-conjugated goat anti-mouse IgG (Sigma) (diluted 1:50 v/v in PBS) for 1 h with rocking. Plates were washed three times with PBS. The images were acquired with an Olympus IX73 microscope (Olympus Corporation, Japan) connected to an Olympus DP73 digital color camera.

## Virus growth curves

BHK cells were incubated with rZB-3A-R127I or rZBatt at an MOI of 0.01 for 1 h at 37°C. Following this infection, the supernatant was removed, the monolayer was washed, and fresh growth medium was added. Samples (in duplicate) were collected at the desired time points (4, 8, 12, 16, and 24 h), and stored at −80 °C. After freeze–thawing three times, virus titer was determined by the 50% tissue culture infective dose (TCID50) method.

## Reverse transcription-quantitative PCR assays

BHK cells were infected with rZB-3A-R127I or rZBatt at an MOI of 1.0 for 1 h. At the indicated time points, samples were collected and total RNA was extracted by RNAiso Plus. Total RNA in each sample was measured using a NanoVue Spectrophotometer. The cDNA was synthesized with TransScript II One-Step gDNA Removal and cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd. China) using a random primer. Finally, real-time PCR assay was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) using a TransStart Green qPCR SuperMix (Beijing TransGen Biotech Co., Ltd. China) and a standard amplification profile with the primers 3D-F (ACTGGGTTTTACAAACCTGTGA, forward primer) and 3D-R (GCGAGTCCTGCCACGGA, reverse primer), to obtain a 107 bp fragment from the 3D region of FMDV. In parallel, reverse transcription-quantitative PCR (RT-qPCR) directed against the 18S rRNA was performed for the RNA loading control using the primers 18S-F (GAGCGAAAGCATTTGCCAAG, forward primer) and 18S-R (GGCATCGTTTATGGTCGGAAC, reverse primer) to obtain a 101 bp fragment. The relative copy number of FMDV was calculated from the $2^{-\Delta\Delta Ct}$ value between 18S rRNA and FMDV RNA.

## Virulence in suckling mice

To compare the virulence between rZB-3A-R127I and rZBatt, 3-day-old BALB/c suckling mice were divided into eight groups (8–10 mice per group), and each mouse was injected subcutaneously into the cervical dorsal area with 100 μL of diluted virus at an MOI of 0.01 to 10×TCID50 as described previously (Zeng J, et al.). The mice in the control group were inoculated with 100 μL of DMEM per mouse. The percentage of surviving suckling mice was recorded every 12 h until 7 days after inoculation.

## Statistical analyses

Data analysis and graphic representation were per-formed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) or Microsoft Excel software programs. Viral growth curves and RNA replication efficiency of rZB-3A-R127I and rZBatt were compared using repeated measures ANOVA. Mouse survival curves were plotted according to the Mantel-Cox method, and survival function across treatment groups was compared using log rank test analyses. $P<0.05$ was considered significant.

## RESULTS

### Rescued viruses from the infectious cDNA clones

Viruses were successfully rescued from the full-length cDNA clone (pZBatt) of ZB/CHA/58(att) and its R127-mutated cDNA clone (pZB-3A-R127I) after *in vitro* transfection in BHK-21 cells. The rescued virus from the pZBatt was designated as rZBatt, and the virus rescued from the pZB-3A-R127I was designated as rZB-3A-R127I. These two viruses could replicate stably and generate typical cytopathic effects (CPEs) in BHK-21 cells. Sequencing of the full-length genomic of rZB-3A-R127I indicated that the nucleotide thymine in the 3A gene was mutated to guanine (T→G) leading to the replacement of the amino acid residue isoleucine (I) in the 3A protein at position 127 with arginine (R), and that there was no additional nucleotide changes in cDNA compared with genomic of rZBatt (Figure 1).

### R127-mutated virus exhibited slightly different plaque size, and reacted similarly with mAb 3E11 in BHK-21 cells

To determine whether the R127 mutation could affect FMDV replication *in vitro*, plaque formations of rZB-3A-R127I and rZBatt were tested in BHK-21 cells. The results showed that the R127-mutated rZB-3A-R127I virus had slightly different plaque formation than the rZBatt virus (Figure 2). However, rZB-3A-R127I and rZBatt in BHK-21 cells had similar reactivity with mAb 3E11 against VP1 of FMDV (Figure 3).

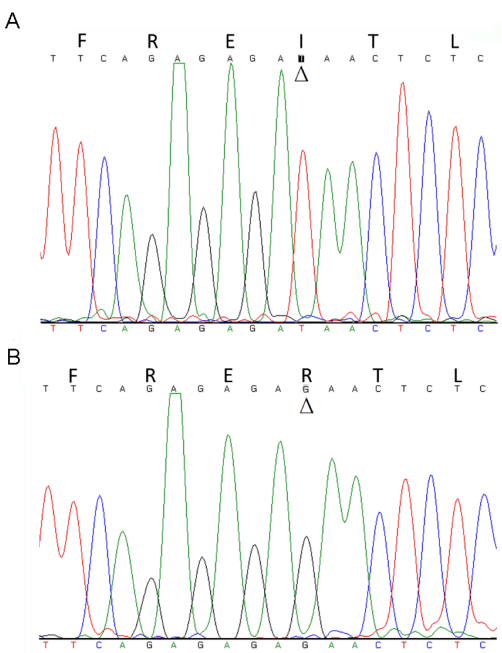


Figure 1. Partial sequence of 3A protein of rescued viruses. Arrows indicate that the nucleotide thymine in the 3A gene was mutated to guanine (T→G) leading to the amino acid residue isoleucine (I) in 3A protein being changed to arginine (R). A: rZBatt; B: rZB-3A-R127I.

### R127-mutated virus exhibited lower replication ability in BHK-21 cells

Viral growth curves of rZBatt and the rZB-3A-R127I in BHK-21 cells were constructed to test whether the amino acid mutation at position 127 of 3A influenced the viral replication ability in cell culture. The results showed that the R127-mutated viral titer was lower than that of rZBatt at the same time points (Figure 4). The trends in the viral growth curves were similar to those observed in the nucleic acid copy numbers, which were quantified by RT-qPCR (Figure 5). Cells infected with rZBatt and rZB-3A-R127I had increased viral RNA copy numbers from 4 h PI, reaching a maximum after 16 h PI. In contrast, viral RNA copy numbers in the cells infected with the rZB-3A-R127I remained lower ($P<0.01$) than that of rZBatt throughout the time course of the experiment. The RT-qPCR results confirmed that the titer of rZB-3A-R127I in BHK-21 cells was lower than that of rZBatt. These results showed that R127 mutation affected the replication ability of the rabbit-attenuated ZB strain.

### R127-mutated virus caused a significantly survival in suckling mice

To compare virulence, 3-day-old suckling mice were inoculated with the rZB-3A-R127I or rZBatt. All mice inoculated with a dose of 10×TCID50 of rZB-3A-R127I or rZBatt died within 2.5 days PI (Figure 6A), and after inoculation with a dose of 1×TCID50, all mice died within 3 days PI (Figure 6B). The survival rate of rZB-3A-R127I-infected mice was significantly higher than that of rZBatt-infected mice at both 10×TCID50 ($P<0.01$) and 1×TCID50 ($P<0.05$). After inoculation with a dose of 0.1×TCID50, the onset of FMD symptoms was delayed, and mice inoculated with rZB-3A-R127I and rZBatt had a survival rate of 37.5% and 25.0%, respectively, at the end of the 7-day observation period (Figure 6C). No significant survival differences were observed between rZB-3A-R127I and rZBatt-infected mice ($P>0.05$). After inoculation with a dose of 0.01×TCID50, no mice died in the R127-mutant and rZBatt virus groups over the 7-day observation period (Figure 6D). All control group mice remained alive during the entire experimental period. The results indicate that the R127 mutation in 3A protein resulted in obviously reduced virulence of the rabbit-attenuated ZB strain in suckling mice.

## DISCUSSION

The virulent ZB strain was responsible for the out-

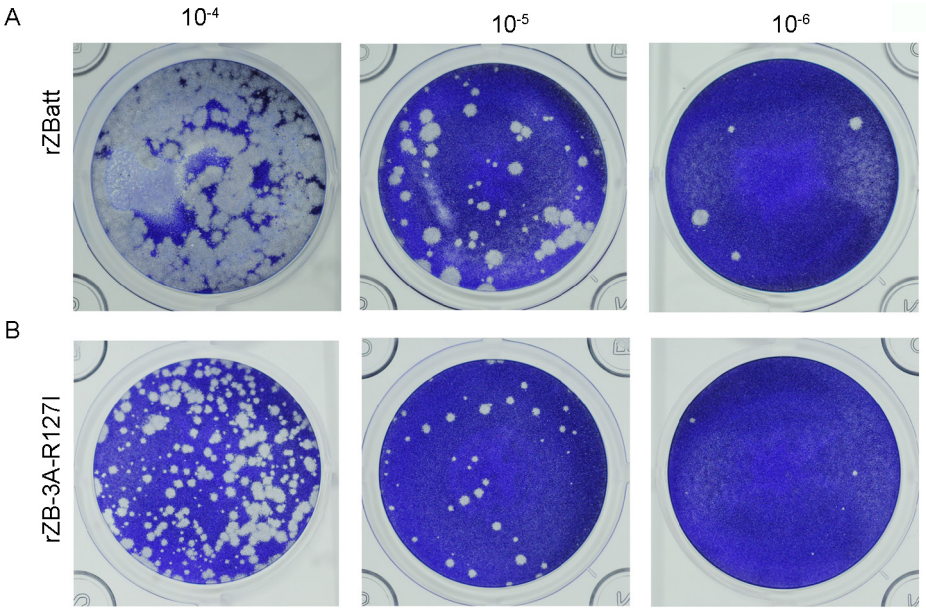


Figure 2. Plaque morphology of rZBatt and rZB-3A-R127I. A: rZBatt; B: rZB-3A-R127I.

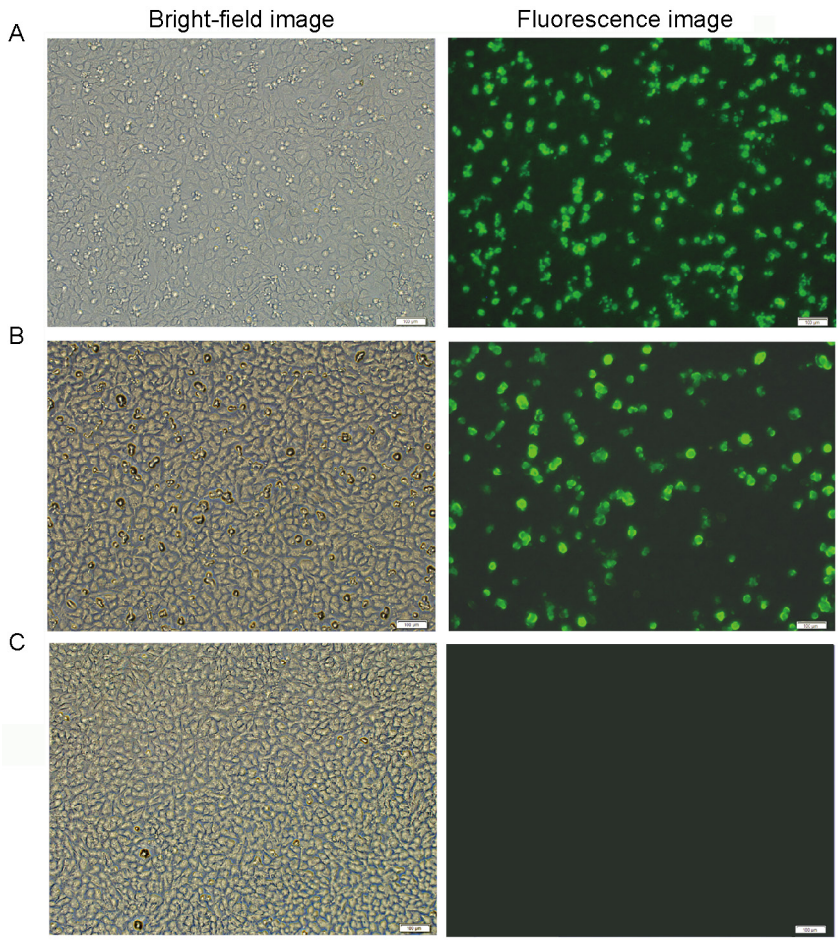


Figure 3. Reactivity of rZBatt and rZB-3A-R127I with mAb 3E11 in an indirect immunofluorescence assay. A: rZBatt-infected BHK-21 cells; B: rZB-3A-R127I-infected BHK-21 cells; C: normal BHK-21 cells as a negative control. Scale bar = 100 μm.

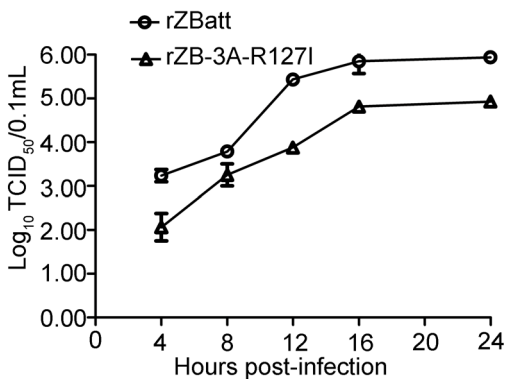


Figure 4. Growth curves of rZBatt and rZB-3A-R127I. BHK-21 cells were infected and harvested at the desired time points post-infection. Growth curves were obtained by titration of the virus present at each time point. Each sample was assayed in duplicate, and values represent the mean±SD of each group.

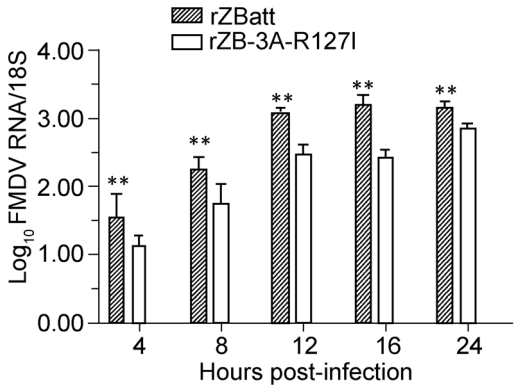


Figure 5. FMDV RNA levels in BHK-21 cells at desired time points post-infection with rZBatt or rZB-3A-R127I, after being normalized to 18S rRNA. For statistical analysis, a one-way ANOVA was used. Statistical significance is indicated by asterisks (**$p$<0.01). Each sample was assayed in triplicate, and values represent the mean±SEM of each group.

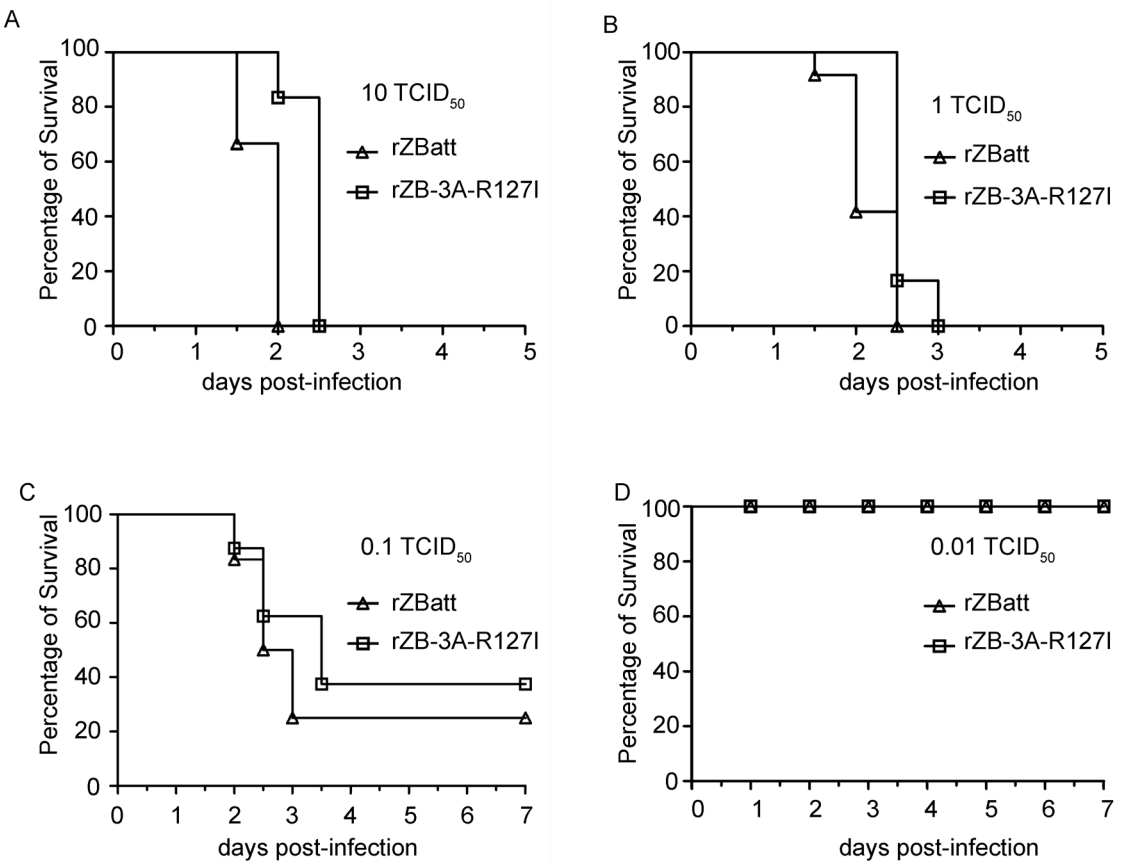


Figure 6. Survival curves of suckling mice inoculated with rZBatt or rZB-3A-R127I (n=8-10 for each group). A: 10×TCID50 per mouse group; B: 1×TCID50 per mouse group; C: 0.1×TCID50 per mouse group; D: 0.01×TCID50 per mouse group. Experiments were carried out twice.

break of FMD serotype Asia1 in China in 1958. In our previous study, we established an infectious cDNA clone of a rabbit-attenuated vaccine strain, ZB/CHA/58(att). Comparative sequence analysis of the virulent and attenuated ZB strains sequences showed that there were five amino acid mutations in the 3A protein after attenuation. The 3A protein played an important role in the host range and pathogenicity of FMDV (Alexandersen S, et al., 2005; Grubman M J, et al., 2004). FMDV C-S8cl adapted to guinea pig resulted in the substitution of residue Q44 to R in the 3A protein (Nunez J I, et al., 2001). Furthermore, mutations in 3A may alter the host range and virulence of FMDV *in vivo* and in cell culture (Knowles N J, et al., 2001). It was reasonable to assume that one of these substitutions in 3A of the virulent ZB strain contributed to the virulence change during the attenuation process. To verify this assumption, we used an infectious clone of ZB/CHA/58(att) as a backbone to generate a plasmid containing the R127 mutation in the 3A protein to explore its impact on the replication, infectivity, and immunoreactivity of the virus. Virus was produced from infectious cDNA by *in vitro* transcription followed by transfection of BHK-21 cells. The virus with the R127 mutation in 3A, rZB-3A-R127I, could be efficiently rescued from BHK-21 cells, showing a similar phenotype to its wild-type rZBatt.

Although the R127-mutated virus and its parental virus showed similar antigenic reactions with mAb 3E11 in an IFA, the slight difference in plaque morphology seen might be an implication of altered replication efficiency and cytopathic effect ability in BHK-21 cells between the mutant and its parental virus. The growth analysis *in vitro* further showed that the R127-mutated virus, with lower replication ability in BHK-21, displayed significant differences in the growth kinetics and RNA replication analysis compared with rZBatt. Moreover, we found that the R127-mutated virus took longer to take effect, as evidenced by the survival time of suckling mice. The results of the *in vitro* growth analysis were consistent with that of the *in vivo* virulence experiments, suggesting that the decreased replication ability of the virus caused by the R127 mutation may contribute to the decreased virulence of rZB-3A-R127I in suckling mice. We believe that the R127 mutation makes the rZB-3A-R127I more charged and less hydrophobic, which would account for the mutant having lower replication ability in BHK-21 cells and reduced virulence in suckling mice. Further studies are needed to further clarify the mechanisms of these changes.

We previously examined the pathogenicity of the virulent ZBCF22 and attenuated ZB/CHA/58(att) strains in suckling mice and guinea pigs. Interestingly, ZBCF22 was less virulent than the rabbit-attenuated ZB/CHA/58(att) in suckling mice. In contrast, ZBCF22 expressed higher virulence than the attenuated strain in a guinea pig model, and the results were very consistent with those observed in virulence experiments in cattle. The virulence discrepancy between the virulent and attenuated strains might be related to the different virus receptor(s) in suckling mice and guinea pig target cells, as suggested previously for FMDV variants (Nunez J I, et al., 2001). Although rZBatt was more pathogenic than rZB-3A-R127I in the suckling mice model, we cannot exclude the possible influence on viral replication and pathogenicity in cattle *in vivo*. Because the 3A protein of FMDV has been found to be associated with host alterations (Beard C W, et al., 2000; Giraudo A T, et al., 1990; Knowles N J, et al., 2001), we also cannot exclude the possibility that the R127 mutation changed the host tropism, which may explain the difference in host tropism of rZBatt and rZB-3A-R127I. Further investigations are needed to elucidate the effects of R127→I on FMDV virulence change in cattle during the attenuation process.

In summary, we have demonstrated the effects of an R127 mutation in the 3A protein on viral replication and infection of an FMDV serotype Asia1 rabbit-attenuated ZB strain by site-directed mutagenesis using a reverse genetics system. Our results suggest that the R127 mutation influenced the replication ability and virulence of the virus. Thus, special consideration should be given to the 3A protein in research on virulence-attenuation relationships and development of an FMDV vaccine. We propose that mutations in the 3A protein of ZB strain are likely to be one of the major determinants of attenuation, and especially that the E78→G, H80→C、K84→N, and R127→I mutations will make the attenuated strain less charged and more hydrophobic than that of virulent parental virus. It would be interesting to examine by reverse genetics how those amino acid substitutions may synergistically or singly contribute to virus attenuation in cattle.

## ACKNOWLEDGMENTS

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## COMPLIANCE WITH ETHICS GUIDELINES

All the authors declare that they have no competing interests. This article does not contain any studies with human or animal subjects performed by any of the authors.

## AUTHOR CONTRIBUTIONS

AG Xin conceived the study, performed the exper-

iments, and wrote the paper. MW Zhu participated in the study design and experiments. Q Hu and HS Miao participated in the virus experiments. ZQ Peng participated in the animal experiments. YW He and L Gao provided the cells. HC Li supervised this study. All authors read and approved the final manuscript.

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