Applied Microbiology and Biotechnology (2022) 106:273–286
https://doi.org/10.1007/s00253-021-11692-2

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Infection and protection responses of deletion mutants of non-structural proteins of foot-and-mouth disease virus serotype Asia1 in guinea pigs

H. Lalzampuia[1] · Subhadra Elango[2] · Jitendra K. Biswal[3] · Narayanan Krishnaswamy[2] · R. P. Tamil Selvan[2] · P. Saravanan[2] · Priyanka Mahadappa[2] · V.Umapathi[4] · G. R. Reddy[2] · V. Bhanuprakash[2] · Aniket Sanyal[2] · H. J. Dechamma[2]

Received: 29 July 2021 / Revised: 9 November 2021 / Accepted: 10 November 2021 / Published online: 10 December 2021

Abstract

The development of a negative marker vaccine against the foot-and-mouth disease virus (FMDV) will enhance the capabilities to differentiate vaccinated from infected animals and move forward in the progressive control pathway for the control of FMD. Here, we report the development of mutant FMDV of Asia1 with partial deletion of non-structural proteins 3A and 3B and characterization of their infectivity and protection response in the guinea pig model. The deleted FMDV Asia1/IND/63/1972 mutants, pAsia$^{\Delta 3A}$ and pAsia$^{\Delta 3A3B1}$ were constructed from the full-length infectious clone pAsia^{WT}, the viable virus was rescued, and the genetic stability of the mutants was confirmed by 20 monolayer passages in BHK21 cells. The mutant Asia1 viruses showed comparable growth pattern and infectivity with that of Asia^{WT} in the cell culture. However, the Asia$^{\Delta 3A3B1}$ virus showed smaller plaque and lower virus titer with reduced infectivity in the suckling mice. In guinea pigs, the Asia$^{\Delta 3A3B1}$ virus failed to induce the disease, whereas the Asia$^{\Delta 3A}$ virus induced typical secondary lesions of FMD. Vaccination with inactivated Asia1 mutant viruses induced neutralizing antibody response that was significantly lower than that of the parent virus on day 28 post-vaccination (dpv) in guinea pigs ($P < 0.05$). Furthermore, challenging the vaccinated guinea pigs with the homologous vaccine strain of FMDV Asia1 conferred complete protection. It is concluded that the mutant Asia$^{\Delta 3A3B1}$ virus has the potential to replace the wild-type virus for use as a negative marker vaccine after assessing the vaccine worth attributes in suspension cell and protective efficacy study in cattle.

Key points

- *Deletion mutant viruses of FMDV Asia1, developed by PCR-mediated mutagenesis of NSP 3A and 3B1, were genetically stable.*
- *The growth kinetics and antigenic relatedness of the mutant viruses were comparable with that of the wild-type virus.*
- *Vaccination of guinea pigs with the deletion mutant viruses conferred complete protection upon challenge with the homologous virus.*

Keywords FMDV 3A and 3B proteins · Deletion mutants · Infectivity

Introduction

India is enzootic for foot-and-mouth disease (FMD) and economic losses due to the disease are more than ₹200 billion/annum (Singh et al. 2013). Currently, India is in stage three of the progressive control pathway for control of FMD and aims to achieve FMD-free status with vaccination by 2030. The success of the FMD control program not only depends on the biannual vaccination of 500 million susceptible livestock such as cattle, sheep, goat, pig, buffalo, yak, mithun, and other wildlife, but also depends on effective sero-surveillance (Paton et al. 2009).

✉ H. J. Dechamma
dechamma.j@icar.gov.in

1 ICAR-NRC On Mithun, Medziphema, Nagaland, India

2 FMD Vaccine Production Laboratory, ICAR-Indian Veterinary Research Institute (IVRI), Hebbal Campus, Karnataka, Bengaluru 560 024, India

3 ICAR-Directorate of Foot-and-Mouth Disease, Mukteswar, Nainital 263138, India

4 FMD Q&C and Q&A Laboratory, Bengaluru, India

Development of gene-deleted modified FMDV vaccine for the serotypes O, A, and Asia1 is important in the context of the development of negative marker vaccines and sero-surveillance in the course of attaining freedom from FMD for India.

Foot-and-mouth disease is one of the viral diseases of livestock affecting all the cloven-hoofed animals. It is caused by the *Aphthovirus* of the *Picornaviridae* family. The virus is quasispecies in nature and undergoes rapid mutation. There are seven antigenically and genetically distinct serotypes and they do not show cross-protection in vaccinated animals (Jamal and Belsham 2013). In India, FMDV O, A, and Asia1 serotypes are reported and antigenically matched strains of all the three serotypes are mandatorily included in the commercial vaccine formulation. The genome is a linear positive-sense single-stranded RNA measuring about 8.5 kb in size encoding four structural proteins (VP1-4) and varying numbers of non-structural proteins (NSP). The 3A NSP of FMDV, a partially conserved protein of 153 amino acids, is involved in the viral replication, virulence, and host range (Gladue et al. 2014). The N-terminal half of the 3A gene encodes for a hydrophilic domain and a membrane-binding hydrophobic domain, which is conserved among the serotypes of FMDV (Knowles et al. 2001). This N-terminal 3A protein is reported to interact with the host signaling pathway and inhibit innate immune response (Li et al. 2016, 2020). Furthermore, the carboxy-terminal half of 3A NSP is not essential for replication in cultured cell lines (Falk et al. 1992; Behura et al. 2016; Li et al. 2020). FMDV is the only *picornavirus* to have three non-identical copies (23 to 24 residues) of the 3B coding region, which is conserved across the serotypes of FMDV. Although a single copy of 3B is sufficient for the generation of new strands of virus wherein the redundant copies are dispensable, the efficiency of RNA replication is maximal when all the three 3B copies are present (Adeyemi et al. 2021). Studies with the deletion of 3A or 3B have been reported that includes 3AB-truncated virus and its companion assay (Bhatt et al. 2018), two marker FMDV vaccine candidates with Lpro and one of the 3B proteins deleted (Uddowla et al. 2012), and an r3AB1-FMDV-NSP vaccine (Jaworski et al. 2011), a virus with the 3AB NSP region deleted as a companion diagnostic assay (Biswal et al. 2015a).

In this study, we deleted the C-terminal of 3A and the complete 3B1 from the vaccine strain of FMDV Asia1/IND/63/1972 ($Asia^{WT}$) and rescued the mutant viruses ($Asia^{\Delta 3A}$ and $Asia^{\Delta 3AB1}$). Genetic stability, antigenic specificity, and growth kinetics of the mutant viruses were compared with that of $Asia^{WT}$. The virulence of the mutant virus, $Asia^{\Delta 3A}$ was comparable with that of $Asia^{WT}$, whereas $Asia^{\Delta 3A3B1}$ lacked pathogenicity in guinea pigs, induced antibody titer, and protected against challenge with the homologous vaccine strain. The polyclonal serum against FMDV 3A peptide did not detect the NSP 3A antigen of $Asia^{\Delta 3A3B1}$ virus. The results indicate that mutant $Asia^{\Delta 3AB1}$ virus is a potential candidate for negative marker vaccine candidate and aid in foolproof monitoring of virus circulation in the susceptible population.

Materials and methods

Cells and viruses

JM109 and TOP 10 *E. coli* cells were used for transformation and propagation of the plasmid. Baby hamster kidney-21 (BHK21 clone 13) cells available in the FMD research laboratory was maintained in Glasgow Minimal Essential Medium (GMEM) with 10% fetal bovine serum, streptomycin (100 mg/L), kanamycin (100 mg/L), and penicillin G (60 mg/L) under 5% CO_2 at 37 °C. Foot-and-mouth disease vaccine virus serotype Asia1/IND/63/1972, maintained at the repository of FMD research laboratory, was used in the study. The cDNA clone ($pAsia^{WT}$) was used for the construction of partial 3A and 3B gene-deleted plasmid constructs and rescue of viable infectious virus (GenBank Accession #AY304994.1) (Saravanan et al. 2011).

Generation of deletion constructs of $pAsia^{\Delta 3A}$ and $pAsia^{\Delta 3A3B1}$

As shown in Fig. 1, the mutation was introduced in the full-length FMDV Asia1/IND/63/1972 infectious cDNA clone ($pAsia^{WT}$) by PCR-mediated mutagenesis using inverse primers (Table 1) using Q5 site-directed mutagenesis kit (NEB, USA) following the manufacturer's protocol with minor modifications. Specific primers were designed flanking the region to be deleted. The amplicons were treated with *Dpn* I enzyme for 2 h and transformed into TOP 10 *E. coli* cells. The ampicillin-resistant clones were grown and plasmid DNA was confirmed by sequencing. The clone $pAsia^{\Delta 3A}$, deleted with 114 bp (38 amino acids) region in the C-terminal half of 3A coding region, served as template DNA for deleting 84 bp (28 amino acids) comprising of 3B1 and part of 3B2 following the above protocol. The clone, $pAsia^{\Delta 3A3B1}$, was confirmed by nucleotide sequencing.

RNA synthesis in vitro, *transfection, and virus recovery*

The full-length infectious clone $pAsia^{WT}$, deleted mutant plasmids, $pAsia^{\Delta 3A}$, and $pAsia^{\Delta 3A3B1}$ were linearized with *Not* I enzyme and *in vitro* transcribed by MegaScript T7 kit (Invitrogen, USA). The synthetic RNA (2 µg) and Lipofectamine 2000 (5 µL) were mixed in 50 µL of Opti-MEM and transfected into the BHK21 cells in 12 well tissue culture plate following the manufacturer's protocol.

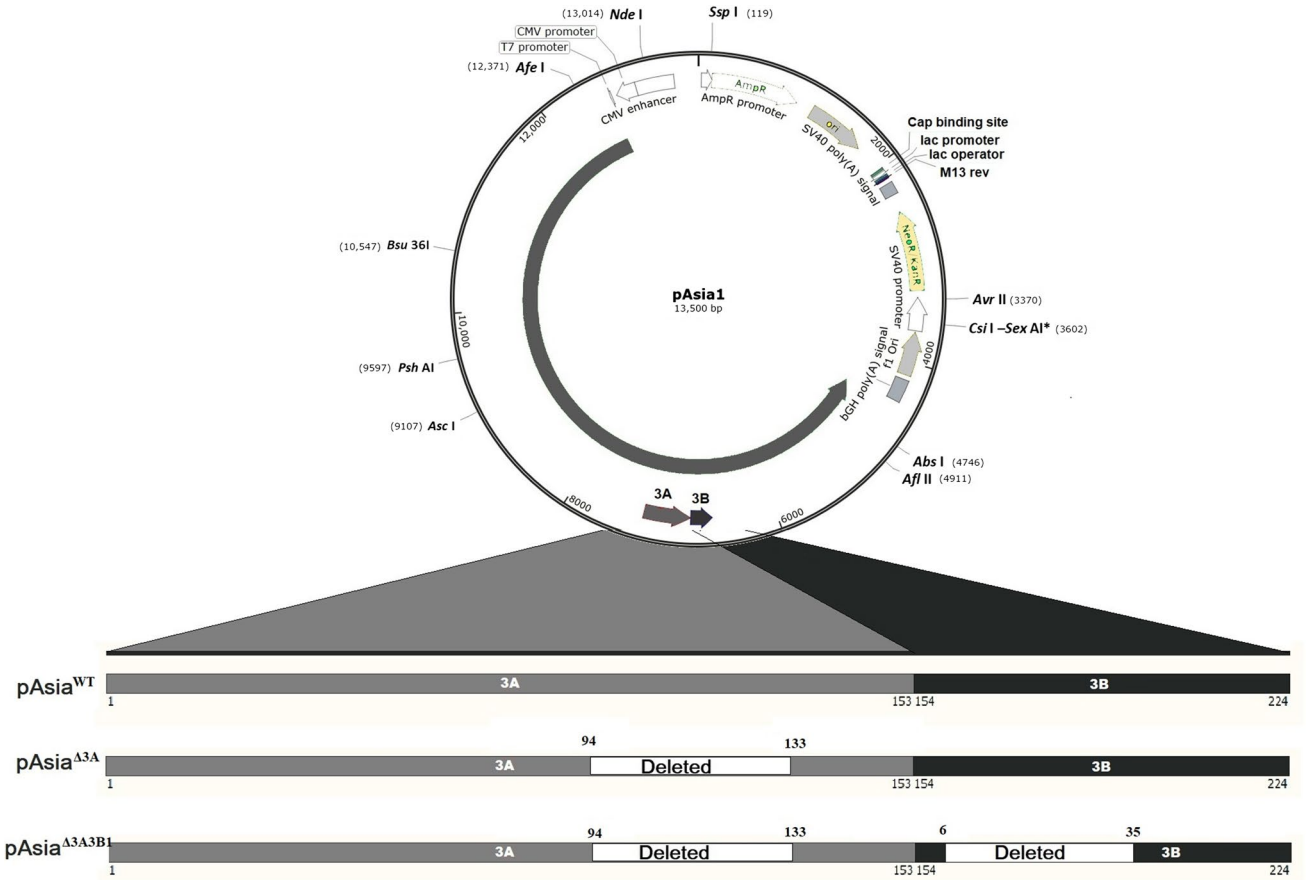

Fig. 1 Schematic representation of the construction of gene-deleted foot-and-mouth disease virus (FMDV) serotype Asia1/IND/63/1972. In the pAsia$^{\Delta 3A}$ plasmid construct, 38 amino acids were deleted in the C-terminal 3A region, keeping the junction of 3A and 3B undisturbed. The plasmid DNA of pAsia$^{\Delta 3A}$ served as a template for deleting the 84 bp of 3B1and part of 3B2 (28 amino acids) to generate pAsia$^{\Delta 3A3B1}$. Six amino acids at the junction of 3A3B1 complement for the 3B2 in pAsia$^{\Delta 3A3B1}$

Table 1 List of oligonucleotide primers used in the study: primers were designed with reference to the FMDV Asia 1 (Gene Bank-AY304994.1)

Primer	Sequence(5' to 3')
Primers used for inverse PCR	
3A 94R	GATGTACTCACTCACTGCGTCATC
3A 133F	AAGGTGAGTGATGACGTGAACTC
3B5689R	ACGCTCAAGTGGCCCGGC
3B5707 F	GTGAAAGCAAAAGCCCCGGTC
Primers used for negative-strand PCR	
L463F	ACCTCCAACGGGTGGTACGC
DHP9	GACCTGGAGGTTGCGCTTGT
NK61R	GACATGTCCTCCTGCATCTG
Primers used for sequencing including flanking sequence of the deleted region	
2C 5051F	TGATGTAAGTGCCAAGGACGGG
QRT3CR	TCTGCCGTCCAACATGATCT

After a short incubation with shaking, 360 µL of Opti-MEM was added and incubated for 4 h, and then, GMEM (2% FBS) was added and kept for incubation in a 5% CO_2 incubator. After 48 h, the cells were freeze-thawed and half of the clarified supernatant was used for infecting the BHK21 cell monolayer. Subsequent five to six blind passages were carried out till FMDV-specific cytopathic effects were observed within 16 h post-infection (hpi). The

rescued virus was amplified in a 175 cm^2 flask, and the aliquots were stored at – 70 °C. The viruses were titrated as reported earlier (Reed and Muench 1938) and the concentration was expressed in $log_{10}TCID_{50}$/mL.

Viral RNA extraction, cDNA synthesis, and DNA sequencing

The viral RNA was extracted from cell culture supernatants using TRIzol (Life Technology, Invitrogen, USA) as described by the manufacturer. The cDNA was synthesized using oligod(T_{17}) using M-MuLV reverse transcriptase (NEB, USA). The cDNA was amplified by PCR using 2C 5051F; QRT3CR primers (Table 1); and Q5 PCR master mix. The PCR product was purified using a PureLink PCR purification kit (Thermo Scientific, USA) and the sequence was determined by automatic DNA sequencing (Eurofins, India). For negative-strand synthesis, cDNA was synthesized using virus-specific forward primer L463F and PCR amplification using FMDV Asia1 specific F-DHP9 and NK61R primer (Horsington and Zhang 2007).

Growth and genetic stability of the mutant viruses

The rescued viable mutant virus $Asia^{\Delta 3A}$ and $Asia^{\Delta 3A3B1}$ were propagated till 20 passages in the BHK21 cells and the virus titer was assessed at every fifth passage (Reed and Muench 1938) and nucleotide sequence of the virus for the deleted region and the flanking sequences were determined.

Sandwich ELISA

Serotype specificity of the rescued virus was confirmed by sandwich ELISA (Bhattacharya et al. 1996). Briefly, the ELISA plates were coated with 50 µL of serotype-specific (FMDV type Asia1, O, and A) rabbit anti-146S serum in duplicate. A virus supernatant (50 µL) was added to the wells. Fifty microliters of tracing sera (serotype-specific guinea pig anti-146S serum prepared in the blocking buffer at predetermined dilutions) was added to respective serotype-specific wells and incubated at 37 °C for 1 h. Subsequently, 50 µL of polyclonal anti-guinea pig IgG horseradish peroxidase conjugate (Dako, Denmark) was added followed by the addition of 50 µL of freshly prepared chromogenic O-phenylenediamine hydrochloride substrate, hydrogen peroxide, and incubated for 15 min at 37 °C. The enzymatic reaction was stopped with 1 M H_2SO_4 and read at 492 nm against 620 nm (TECAN Sunrise ELISA plate reader, Life Sciences, Switzerland).

Two-dimensional micro-neutralization test

Two-dimensional micro-neutralization tests were done to check the antigenic relatedness of the $Asia^{WT}$ wild-type virus *vis-à-vis* the mutated viruses. Two-fold dilution of FMDV Asia1 bovine vaccine serum was prepared in GMEM and heat inactivated at 56 °C for 15 min. Diluted serum (50 µL/well) was added to the 96 well tissue culture plate in triplicate to which serially diluted (ten-fold) virus (50 µL/well) was added, mixed, and incubated at 37 °C for 1 h. After incubation, BHK21 cells were added to each well at 0.5×10^5 cells per well and further incubated at 37 °C under 5% CO_2 for 48 h. The plates were then read and the antibody titers of the FMDV Asia1 serum against the mutant and wild-type virus were determined (Reed and Muench 1938). The relationship between the $Asia^{WT}$ virus and its deletion mutants was expressed as the "r1" value (Rweyemamu et al. 1978).

Western blot analysis of the virus proteins

Virus structural capsid proteins and 3A NSP were confirmed by Western blot analysis as described (Sambrook and Russell 2001). Briefly, cell lysate (BHK21 cells infected with 0.1 moi of virus) was collected at 12 hpi and was resolved in 12% SDS-PAGE and the protein bands were electro-transferred onto a 0.22-µm PVDF membrane. The membrane was blocked in PBS-T with 5% skimmed milk powder and incubated at 37 °C for 1 h. The membrane was incubated with FMDV Asia1 146S antigen rabbit antiserum (1:200 dilutions) at 37 °C for 1 h. After three washing cycles of each for 5 min, the membrane was incubated with goat anti-rabbit immunoglobulin HRP conjugate (1:5000 dilutions in PBS-T) at 37 °C for 1 h. After three washes with PBS-T, the blot was developed using chemiluminescent substrate (Clarity Western ECL; Bio-Rad) and visualized under the UViTEC chemiluminescence system. For the detection of FMDV 3A, BHK21 cell lysate collected at 8 hpi (2 moi) was used following the above protocol except for the change of primary antibody and detection system. Rabbit anti-3A polyclonal sera were used at 1:100 dilution. Blot was developed using the chromogen, 3,3′-diaminobenzidine.

Growth kinetics of rescued viruses in BHK-21 monolayer cells

The replication kinetics of the rescued virus was studied in 6-well tissue culture plates with preformed BHK21 cell monolayers. Cells were washed once with serum-free GMEM and infected with 0.01 moi of $Asia^{WT}$, $Asia^{\Delta 3A}$, and $Asia^{\Delta 3A3B1}$ viruses. After an hour of adsorption, the cells were washed with GMEM (pH 7.0) to remove the residual virus and replaced with 2 mL GMEM supplemented with

2% FCS. The plate was incubated at 37 °C in CO_2 tension. At 0, 4, 8, 12, 16, and 24 hpi, virus-infected cell lysate was collected and the titer of the virus of three independent experiments was estimated by $TCID_{50}$ and expressed as $\log_{10}$ $TCID_{50}$/mL (Reed and Muench 1938).

Quantification of the virus by real-time PCR

Viral RNA quantification was done using FMDV VP1-specific primers (QRT 1D F: CGGTCCGCGACGTACTAC TT; QRT 1D R: GCGGGTGATGGGTTGCTTCT). RNA isolation (QIAamp kit, Qiagen, USA) and cDNA synthesis (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, USA) were done. Quantification was done using SYBR green master mix (Applied Biosystems, Thermo Fisher Scientific, USA) by standard curve method, using a serial dilution of a known amount of $pAsia^{WT}$ plasmid. Cells without virus infection served as no template control. Three experimental replicates were generated and each sample was tested in triplicate in MicroAmp Optical 96-well reaction plate (Applied Biosystems, Thermo Fisher Scientific, USA) in 10 µL total reaction volume.

Plaque assay

The plaque size and morphology of the parent virus and the mutated viruses were studied in preformed monolayer BHK21 cells in 35 mm (Corning) tissue culture dishes. The monolayer was infected with serial dilutions ranging from 10^{-1} to 10^{-6} of the virus stock in duplicate. After an hour of incubation, cells were washed twice with GMEM (pH 7.0), were overlaid with 3 mL of plaquing media (equal volume of serum-free GMEM and 3% low melting point agarose kept at 42 °C), and incubated at 37 °C for 48 h and stained with 0.1% crystal violet (w/v) in 10% formaldehyde (v/v) in PBS (Bachrach et al. 1957). The diameters of ten randomly selected plaques were measured using NIS software (NIS Elements BR H.20.03, Nikon, Tokyo) under an inverted microscope to calculate the mean diameter of plaque.

Particle stability thermal release assay (PaSTRy)

Capsid stability of the wild and mutant viruses was performed in 96 well PCR plates using 7500 Fast Real-Time PCR Machine (Applied Biosystem). Virus capsid particles were inactivated, purified by cesium chloride gradient, and dialyzed against TEN buffer (pH 7.4) and the capsid particles were used at a concentration of 500 ng (3 µL). Diluted SYBR Green-II dye in TEN buffer 15 µL (1:100) was mixed with virus capsid particles and the volume was made to 30 µL in the Tris–EDTA buffer. The reaction was initiated with the temperature ramp from 25 to 99 °C in 1 °C increment at an interval of 10 s (1%). SYBR Green-II fluorescence was detected at excitation and emission wavelengths of 490 nm and 516 nm, respectively. The release of RNA from the dissociated capsids was detected by an increase in the fluorescence signal. The melting temperature was taken as the negative first derivative of the fluorescence curve (Walter et al. 2012).

Transmission electron microscopy (TEM)

Aliquots of inactivated, purified by cesium chloride gradient, and dialyzed against TEN buffer (pH 7.4) $Asia^{WT}$ and mutant viruses were diluted to 0.1 mg/mL (5 µL), layered onto Formvar/carbon-coated copper grids (FCF 300-CU; Electron microscopy sciences, USA). The excess sample was washed with Milli-Q water and stained with 1% uranyl acetate for 90 s, the excess stain was blotted away with filter paper and the grids were allowed to dry. The grids were examined on TEM (HT7700 series operated at 60 kV; camera XR81B, 3296X2464 pixels).

Virus infectivity studies in suckling mice and adult guinea pigs

Animal experiments were carried out as per the guidelines of the governing body. Institute animal ethics committee (IAEC) approval was obtained to conduct the studies in the laboratory animals (F No.8–56/Vol II/RCSS/2018–19/2). Infectivity study was carried out with live virus in the suckling mice and guinea pigs, whereas immunogenicity experiment was performed with a vaccine prepared from the inactivated virus in guinea pigs.

Suckling mice

Suckling mice (3 to 5 days old) of Albino Swiss breed ($n = 6$/group/passage) were randomly divided into four groups and administered 100 µL of 100, 10, and 1 $TCID_{50}$/mice of $Asia^{WT}$, $Asia^{\Delta 3A}$, and $Asia^{\Delta 3A3B1}$ virus (diluted in GMEM) by intraperitoneal route. The control group was mock injected with GMEM. The mice were observed for death at 24-h intervals for 72 h and the survivors were sacrificed. Keeping death as endpoint observation, 50% lethal dose (LD_{50}) was determined (Walter et al. 2012). The skeletal muscles of dead or sacrificed mice were homogenized in PBS (pH 7.4) or stored in 50% glycerol-PBS. The tissue homogenate was clarified by centrifugation, filtered through 0.45-µm filters, and then used for inoculation of BHK21 cells or injection of suckling mice (Baranowski et al. 2003).

Total RNA was extracted from each passaged tissue sample using TRIzol (Invitrogen) and cDNA was synthesized. Negative-strand PCR was done by serotype-specific NK61R (5' GACATGTCCTCCTGCATCTG 3') and DHP9 (5' GAC

CTGGAGGTTGCGCTTGT 3') primers to confirm replication of the virus in mice. The nucleotide sequence of the deleted and the adjoining region of the genome was analyzed by Sanger's sequencing. Furthermore, tissue homogenate from the second passage (500 µL) was used for infecting the BHK21 cells in 12-well tissue culture plates to confirm the recovery of viable virus from the mice. After the adsorption time for 1 h at 37 °C, virus inoculum was replaced with serum-free GMEM media and incubated for 16 h to observe for cytopathic effect.

Guinea pig

FMDV 1X10^5 $TCID_{50}$ of Asia^{WT}, Asia$^{\Delta 3A}$, and Asia$^{\Delta 3A3B1}$ were administered intradermally in the right hind footpad (300 µL/animal) in the Dunken Hartley guinea pigs of 12–16 weeks age ($n = 4$/group/passage) and observed at 24-h interval for 7 days for the presence of primary and secondary lesions. The appearance of vesicles at the site of inoculation (right hind footpad), defined as a primary lesion, was invariably recorded at every passage. The appearance of vesicles in other footpads was defined as a secondary lesion that was scored on a scale of 1–4. Score four was given when fulminating secondary lesions were recorded. Vesicular fluid and infected tissue collected from the affected area between days 3 and 7 in PBS (pH 7.4) served as inoculum for the subsequent seven passages (Núñez et al. 2007). Sera collected on 30 dpi were used for demonstrating FMDV Asia1-specific antibodies in guinea pigs by liquid-phase blocking ELISA (LPBE) (Hamblin et al. 1986).

Immune response studies in guinea pigs

Vaccine preparation

The vaccine was prepared from the purified Asia^{WT} and mutants Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ 146S antigen by standard procedure with minor modification (Ganji et al. 2018). Briefly, the wild-type and mutant FMDV Asia1 viruses were bulk produced in BHK-21 cells in roller bottles, collected after observing the cytopathic effect, clarified by centrifugation at 3000 × g for 30 min, and inactivated with binary ethyleneimine 3 mM at 37 °C for 24 h. The inactivated viruses were concentrated with 8% polyethylene glycol 6000 (w/v). Purification of the inactivated viral antigen (146S) was done by the cesium chloride density gradient ultracentrifugation technique and the concentration of 146S particle from the centrifuged fractions was determined by spectrophotometry. The required dose of antigen was diluted in PBS (pH 7.4) and mixed with montanide ISA-201 (Seppic, France) at 50:50 ratio (w/w), homogenized for 1 min (twice with a break of 30 s). The prepared vaccine was stored at 4 °C till use.

Immunization and challenge

A total of 32 guinea pigs of either sex at 12–16 weeks of age were divided into the following four groups ($n = 8$/group): G1 received a vaccine containing Asia^{WT}, G2 received a vaccine containing Asia$^{\Delta 3A}$, G3 received a vaccine containing Asia$^{\Delta 3A3B1}$, and G4 represented negative control and received PBS (pH 7.4). Groups G1, G2, and G3 were administered with 1 µg (200 µL/animal) of virus antigen intramuscularly in the thigh region. On day 30 post-vaccination (dpv), the animals were challenged with 100$GPID_{50}$ (50 µL) intradermally in the right hind footpad and was observed for primary and secondary lesions for the next 7 days. Animals not developing the secondary lesions were considered as protected to calculate the percent protection. The blood was collected on 0, 7, 14, and 28 dpv and the serum was used for determining the neutralizing antibody titers. Sera were heat inactivated at 56 °C for 30 min and was used for the standard VNT assay (OIE 2018) on BHK21 cells using FMDV Asia1/IND/63/1972.

Statistical analysis

Multistep growth kinetics of the Asia^{WT}, Asia$^{\Delta 3A}$, and Asia$^{\Delta 3A3B1}$ at different hpi on the titer (Log_{10} $TCID_{50}$/mL) and viral RNA concentration (µg/mL) were analyzed by two-way repeat measures ANOVA with Bonferroni *post hoc* test. Each point in the line chart represents the mean ± standard error of three experiments. For quantifying the viral RNA, each sample was analyzed in duplicate. The effect of propagation of the three different Asia1 virus mutants till 20 passages on the virus titer was analyzed by two-way ANOVA with Bonferroni *post hoc* test. Infectivity of the Asia1 virus mutants at 100, 10, and 1 $TCID_{50}$/mL in the suckling mice ($n = 6$/virus type) was analyzed by the Kaplan–Meier survival curve keeping death or survival as endpoint event at 72 hpi. A log-rank test was used to find the difference in the mortality of suckling mice among the Asia1 virus types. The secondary lesion score (1 to 4) in guinea pigs induced by three virus types that was recorded till passage 7 was analyzed by the Kruskal–Wallis test with Dunn's *post hoc* test. The effect of vaccination with three Asia1 virus types on the neutralizing antibody titer on 7, 14, and 28 dpv was analyzed by two-way repeat measures ANOVA with Bonferroni *post hoc* test. The results were shown as mean ± standard error ($n = 8$) and the mean difference was considered significant when $P < 0.05$. GraphPad Prism 5.0 was used for the statistical analysis and generation of line and bar charts.

Results

Recovery of the FMDV Asia^{WT} and its deletion mutants

Nucleotide sequence of the plasmid constructs of pAsia^{WT}, pAsia$^{\Delta 3A}$, and pAsia$^{\Delta 3A3B1}$ confirmed the correct orientation of the structural and NSP coding regions of FMDV. The mutant clone pAsia$^{\Delta 3A}$ was confirmed for the deletion of C-terminal 3A-94 to 133 amino acids (114 bp), while pAsia$^{\Delta 3A3B1}$ was confirmed for the deletion of C-terminal 3A-94 to 133 amino acids (114 bp) and 3B 7 to 34 amino acids (84 bp). The *in vitro* transcribed RNA was transfected in BHK21 cells and serial blind passage of Asia^{WT} virus up to 3 monolayer produced cytopathic effects. In the fourth passage, cytopathic foci were observed for BHK21 cells for mutants Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ virus. Furthermore, at passages five and six, the cytopathic effect was observed at 12–16 hpi and an increase in the virus titer was also seen. Virus replication was confirmed by amplification of the VP1 region of the FMDV negative-strand genome, by negative-strand PCR, which amplified ~ 459 bp (Figure S1). Finally, nucleotide sequencing (1100 bp; C-terminal 2C to N-terminal 3C) confirmed deletion of 114 bp of 3A gene in Asia$^{\Delta 3A}$ and 114 bp of 3A gene and 84 bp of 3B gene in Asia$^{\Delta 3A3B1}$.

Characterization of the deletion mutant viruses

Antigenic specificity and identity

Serotype typing ELISA confirmed that the Asia^{WT} and its rescued mutant viruses reacted only with FMDV Asia1 antiserum indicating its specificity (Fig. 2a). The antigenic relatedness of the Asia1 mutant viruses with that of the parent virus, as assessed by two-dimensional neutralization tests, revealed that the r1 value of Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ was 0.90 ± 0.022 and 0.69 ± 0.01, respectively (Fig. 2b) and indicated that the mutant viruses were antigenically closely related with that of wild-type FMDV Asia1.

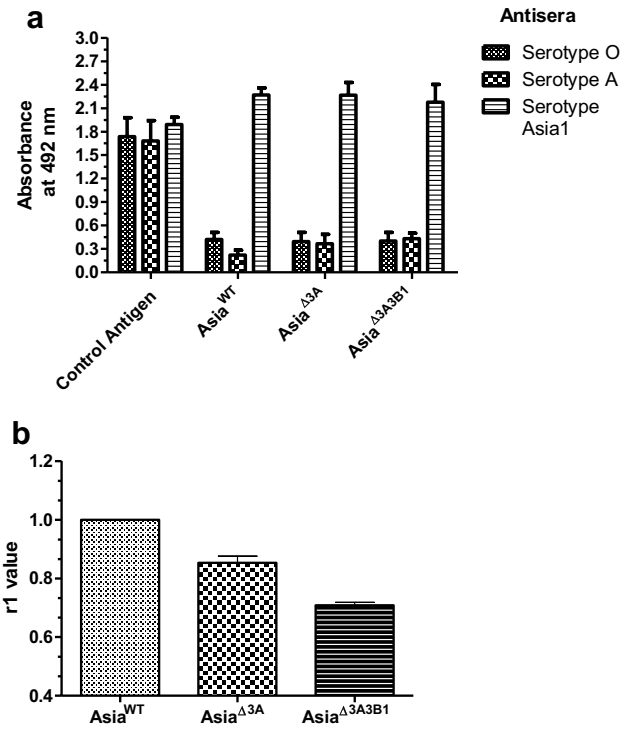

Fig. 2 Antigen specificity and relatedness of the recovered recombinant foot-and-mouth disease virus (FMDV) Asia1 mutant viruses. a Demonstration of the serotype specificity of Asia1 mutant viruses using serotype differentiating sandwich ELISA at monolayer passage number six. Absorbance was recorded at 492 nm. **b** Two-dimensional micro-neutralization test ($n = 3$) showing that Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ mutant viruses were antigenically related to that of Asia^{WT}

Growth kinetics, replication, and plaque formation efficiency of the FMDV Asia mutant viruses

Multistep growth kinetics

The growth of mutant viruses showed a comparable pattern of replication of the wild-type virus and the mutant viruses over a period of 24 hpi. As expected, an extremely significant time-dependent increase in the virus titer was observed for all the three virus types ($P < 0.001$). Peak titer was recorded at 12–16 hpi, while the rate of increase in the titer was between 4 and 8 hpi. However, a significant difference between the parental virus and mutant viruses (Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$) was observed such that the virus titer ($\log_{10}$TCID$_{50}$/mL) of Asia^{WT} was significantly high at 8, 12, 16, and 24 hpi ($P < 0.05$) as compared with the mutant viruses (Fig. 3a).

Kinetics of viral RNA synthesis of the mutant viruses

Kinetics of the viral RNA synthesis of the mutant and wild-type viruses in BHK21 cell monolayer infected with 0.01 moi showed a similar pattern of RNA synthesis to that of parental virus (Fig. 3b). Furthermore, the time-dependent increase in the concentration of viral RNA was comparable with that of the virus titer. The concentration of viral RNA was peak at 12 hpi and it was higher in the Asia^{WT} than Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ ($P < 0.05$).

Virus titration

The titers of the viruses ($\log_{10}$TCID$_{50}$/mL) during bulk production were 6.7 for the wild type, 6.2 for Asia$^{\Delta 3A}$ and

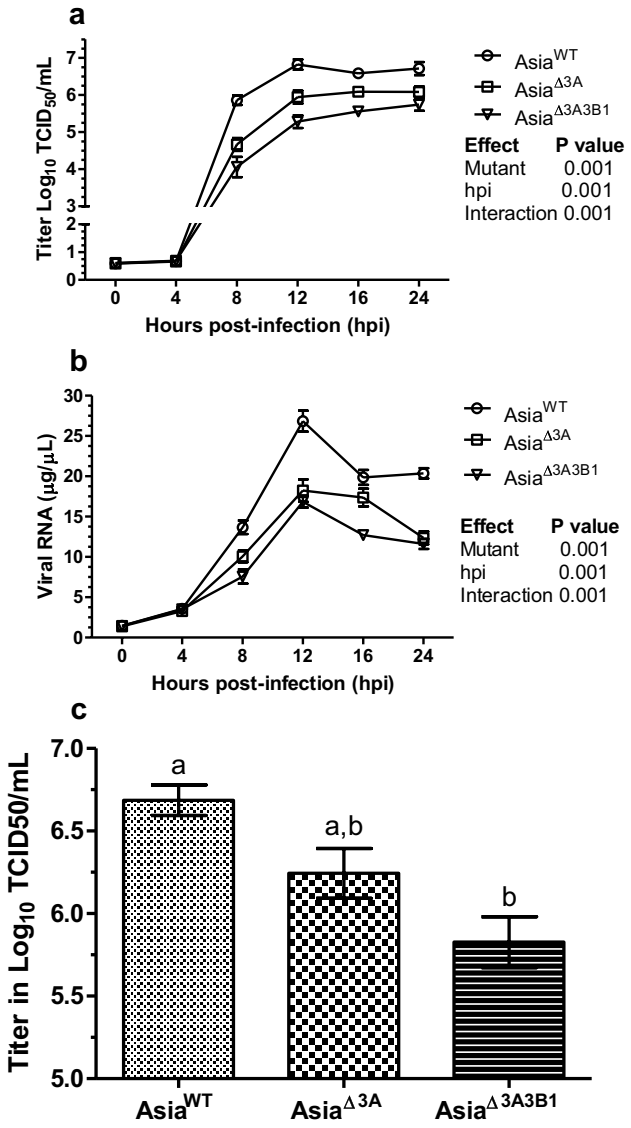

Fig. 3 Replication characteristics of the foot-and-mouth disease virus (FMDV) Asia1 rescued mutant viruses. a Multistep growth curve analysis of FMDV Asia1 wild-type virus and its deletion mutants at passage six. A significant increase in the virus titer ($log_{10}TCID_{50}$/mL) of Asia^{WT} was recorded at 8, 12, 16, and 24 hpi as compared with the mutant viruses ($P < 0.05$) **b** Concentration of the FMDV Asia1-specific RNA at different hpi in the BHK21 cells at passage six. A significant increase in the viral RNA of Asia^{WT} was recorded at 8, 12, 16, and 24 hpi as compared with the mutant viruses ($P < 0.05$). For real-time PCR, each sample was analyzed in triplicate. **c** Effect of mutations on the virus titer following bulk production. Bars with different superscripts differ significantly ($P < 0.05$). Each data point in the bar chart represents the mean ± standard error of three experimental replicates

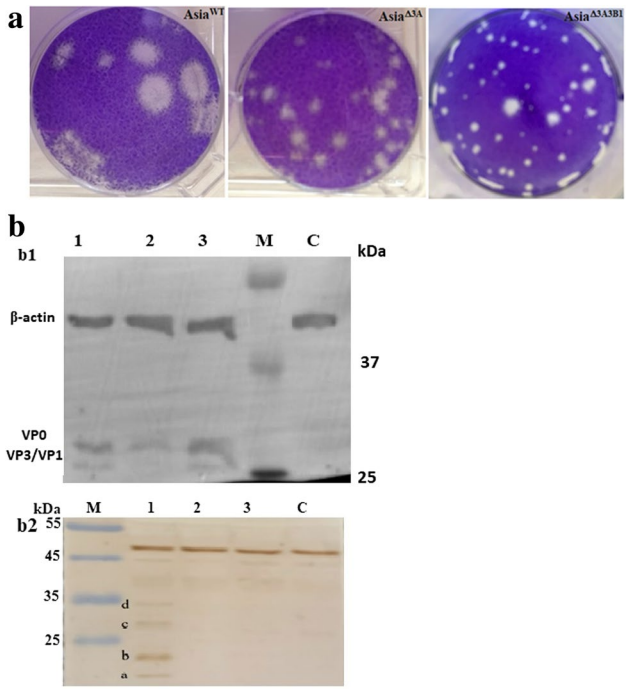

Fig. 4 Characterization of the rescued foot-and-mouth disease (FMD) Asia1 mutant viruses. a) Formation of plaques in BHK21 monolayer cells indicates cytopathic effect and the diameter (mm) of the plaque was 5–7 for Asia^{WT}, 3–5 for Asia$^{\Delta 3A}$, and 2–5 for Asia$^{\Delta 3A3B1}$. **b1)** Demonstration of the structural proteins of FMDV Asia1 by Western blot. Lanes 1–3 represent Asia^{WT}, Asia$^{\Delta 3A}$, and Asia$^{\Delta 3A3B1}$ viruses, respectively. Lanes M and C represent molecular marker and lysate of mock-infected cells, respectively. **b2)** Demonstration of the non-structural protein 3A of FMDV by Western blot. Lanes M and C represent molecular marker and lysate of mock-infected cells, respectively. Lanes 1–3 represent Asia^{WT}, Asia$^{\Delta 3A}$, and Asia$^{\Delta 3A3B1}$ viruses, respectively. Band designation of lane 1: a-3A; b-$3AB_1$; c-$3AB_1B_2$; d-$3AB_1B_2B_3$. gene-deleted foot-and-mouth disease virus **c)** Representative negative-stained transmission electron micrographic images of the capsids of Asia^{WT}, Asia$^{\Delta 3A}$, and Asia$^{\Delta 3A3B1}$ viruses with a size of ~ 25 nm. **d)** Particle stability thermal release assay (PaSTRy): representative images indicate the melt curve of Asia$^{\Delta 3A}$, Asia$^{\Delta 3A3B1}$, and Asia^{WT} (left to right). The negative first derivative of the fluorescence curve was considered as melting temperature. The Asia^{WT} virus showed capsid dissociation at 39 °C, which was not observed in capsids of mutant viruses

5.8 for Asia$^{\Delta 3A3B1}$ (Fig. 3c). Overall, less than 1 log_{10} difference in the titer was observed between Asia^{WT} and its deletion mutants. The results of the plaque assay supported the results of virus titers. The parent Asia^{WT} produced larger plaques of 5 to 7 mm, while the mutant Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ produced plaques of 3 to 5mm diameter. The mutant virus Asia$^{\Delta 3A3B1}$ exhibited the smallest plaque phenotype of about 3 mm (Fig. 4a) and produced a tenfold lower virus titer than the wild-type virus. These results indicated that the deletion of NSP of FMDV such as 3A and 3B1 from Asia^{WT} did not abolish the replication; however, it decreased the efficiency of virus replication.

Immunoreactivity

The FMDV Asia1 146S hyper-immune serum detected the capsid proteins VP1/VP3 and VP0 of the wild-type virus and

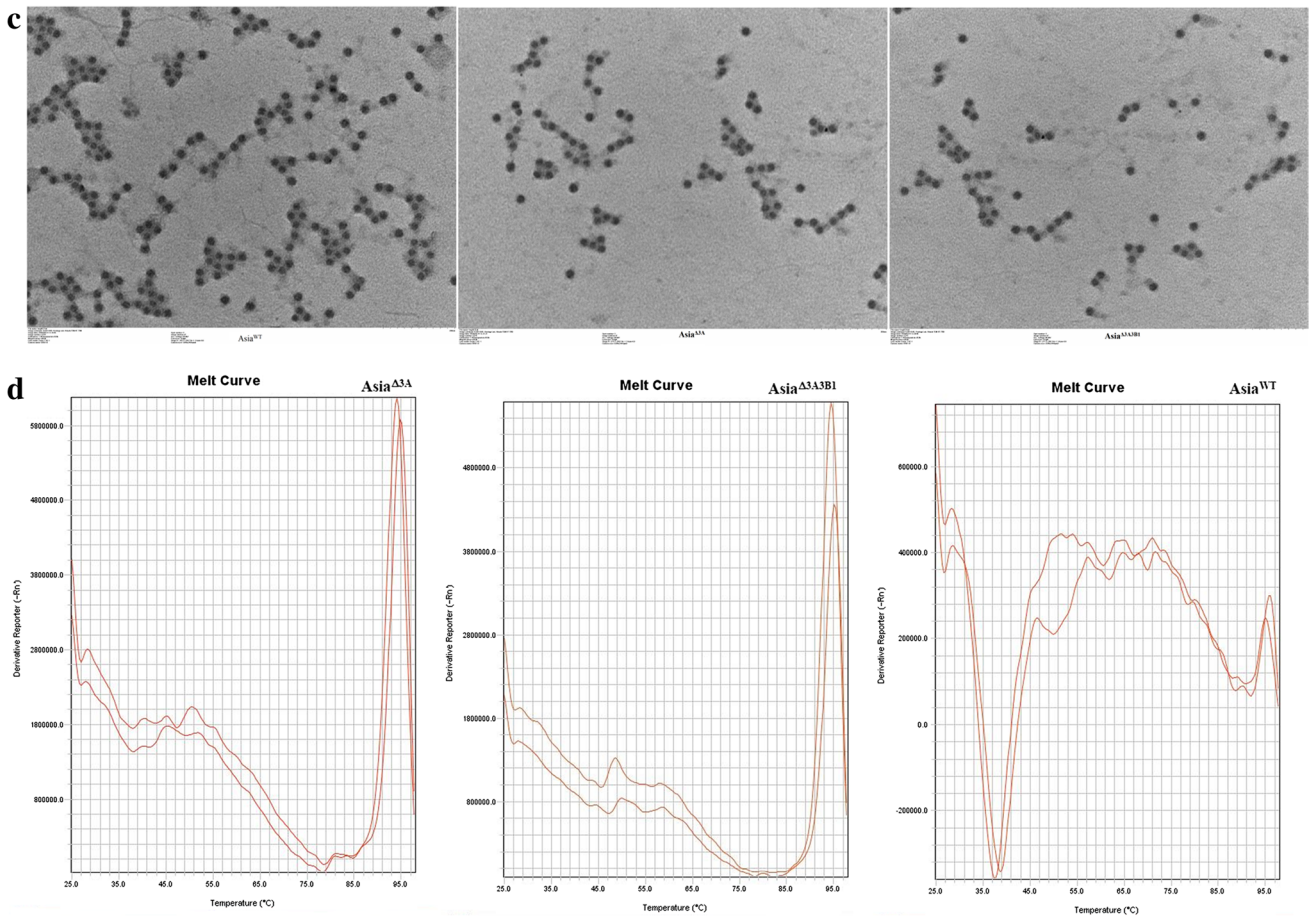

Fig. 4 (continued)

the Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ viruses. However, a decrease in the band intensity of the 3A deletion mutant virus was observed (Fig. 4b1). FMDV 3A peptide polyclonal antiserum reacted only with wild-type Asia1 virus showing varying mass protein bands a, b, c, and d, which are the precursors of NSP 3AB (Fig. 4b2).

Characterization of the mutant virus capsids by TEM and particle stability thermal release assay (PaSTRy)

In order to compare the size of the capsids produced by the deletion mutants, negative-stained transmission electron micrograph images were captured. Viruses of Asia1 wild-type and mutant capsids measured about 25–30 nm. Capsids appeared similar in size, but differed in the number of capsids per unit area. Asia$^{\Delta 3A3B1}$ virus showed half the number of virion capsids as compared with the parent virus (Fig. 4c). Assessment of capsid stability by PaSTRy revealed that both the mutant viruses did not show clear dissociation of capsid followed by an increase in the fluorescence. Instead, very small peak was observed for Asia$^{\Delta 3A}$ at 37 °C that was absent in Asia$^{\Delta 3A3B1}$. The Asia^{WT} virus showed clear capsid dissociation at 39 °C followed by a peak rise in the fluorescence. This indicated that the virus genome was loosely encapsidated and dissociation of capsid did not lead to the release of RNA and increase in the absorbance (Fig. 4d).

Genetic stability of mutant viruses

The rescued mutant viruses (Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$) genome stability for the deleted gene was studied by analyzing nucleotide sequencing. The virus genome amplified from serial monolayer passages 1, 5, 10, 15, and 20 showed deletion of the target regions of 3A and 3B1 and remained stable (Figure S2). The initial virus titer, which was 6.2 $\log_{10}$TCID$_{50}$ for Asia$^{\Delta 3A}$ and 5.8 $\log_{10}$ TCID$_{50}$ for Asia$^{\Delta 3A3B1}$, showed an overall reduction of 0.4 to 0.2 $\log_{10}$ by passage 20 (Figure S3).

Infectivity studies in the suckling mice and guinea pigs

Infection of the suckling mice with Asia^{WT} and its deletion mutants at 1 TCID$_{50}$ did not affect the survival. However, at 100 TCID$_{50}$, all the suckling mice in the Asia$^{\Delta 3A}$ group died

at 48 hpi and the animals manifested symptoms like tremors, ataxia, and paralysis of the hind limbs, while complete death was recorded at 72 hpi in the $Asia^{\Delta 3A3B1}$ group ($P = 0.024$) and group infected with $Asia^{WT}$ died by 24 hpi (Fig. 5a). Inoculums of 1.25 $\log_{10}TCID_{50}$ of wild and mutant $Asia^{\Delta 3A}$ virus produced 50% lethality, whereas 10 $\log_{10}TCID_{50}$ inoculums of $Asia^{\Delta 3A3B1}$ produced 50% lethality in mice. Infection of guinea pigs with $Asia^{WT}$ and $Asia^{\Delta 3A}$ viruses induced appreciable secondary lesions by passages 3 to 4. Though the severity of the primary lesions showed a similar trend with wild-type virus, the secondary lesion score in the $Asia^{\Delta 3A}$ group was significantly lower than that of $Asia^{WT}$ ($P = 0.024$). However, the $Asia^{\Delta 3A3B1}$ virus did not induce appreciable secondary lesions, which might be due to the failure of virus replication or inability to induce viremia in guinea pigs (Fig. 5b).

Protective efficacy of the inactivated vaccines prepared from the $Asia^{WT}$ and its deletion mutants in guinea pigs

Vaccinated animals developed a significant increase in FMDV-specific neutralizing antibodies from dpv14 to 28 ($p < 0.0001$). However, unvaccinated animals did not elicit detectable FMDV-specific antibodies. On dpv 7, the neutralizing antibody titer did not differ significantly among the three types of Asia1 viruses. However, a significantly low ($P < 0.05$) antibody titer of $Asia^{\Delta 3A}$ and $Asia^{\Delta 3A3B1}$ virus was recorded as compared with $Asia^{WT}$ on dpv 14 and 28 (Fig. 6a). After the challenge with virulent homologous wild-type virus, all the vaccinated animals were protected and did not show clinical lesions of FMD, whereas unvaccinated control animals showed primary and secondary lesions such as vesicles in both hind and forelimbs (Fig. 6b). The result demonstrated that the deletion mutant viruses' vaccine is potent as that of inactivated wild-type virus vaccine against clinical infection in spite of low FMDV-specific antibodies.

Discussion

Control of FMD in enzootic regions is widely practiced by biannual vaccination with the whole-virus inactivated vaccines. The current chemical inactivated vaccine is helpful in reducing the incidence of new outbreaks, but has many limitations. Some of the major concerns are the use of virulent FMDV in large manufacturing units and challenges in the removal of NSP residuals to make the vaccine DIVA (differentiating infected from vaccinated animals) compatible. To overcome the limitations associated with the removal of NSP in FMD vaccines, a number of novel vaccine platforms are tried, of which gene-deleted negative marker vaccine with DIVA capability may be the suitable choice for successful implementation of the FMD control program. The virulent genomic region deleted viruses can replace the current virulent vaccine strain of FMDV as well as enable sero-surveillance of the vaccinated population.

In this study, the genomic region corresponding to 38 amino acids of C-terminal 3A and 28 amino acids of 3B1B2 NSP were deleted from FMDV Asia1/IND/63/1972 vaccine strain cDNA clone. The construction of deletion mutants was aided by PCR-mediated mutagenesis using inverse primers (Kunkel 1985). The viable virus was recovered and characterized for serotype specificity in sandwich ELISA, which reacted specifically with Asia1 serotype antisera. Both the mutant viruses ($Asia^{\Delta 3A}$ and $Asia^{\Delta 3A3B1}$) showed antigenic relatedness to the parent wild-type virus. The growth pattern of the wild and mutant viruses was comparable; however, the virus titer of the deletion mutants showed a reduction of 0.5–1.0 $\log_{10}TCID_{50}$ compared with $Asia^{WT}$. Supporting the virus titer results, both the deletion mutants $Asia^{\Delta 3A}$ and $Asia^{3A3B1}$ produced smaller size plaque phenotypes indicating less efficient growth of the deletion mutant viruses as compared with wild-type virus, which produced larger plaques. Similarly, wild-type virus titer and quantity of RNA were highest at 12 hpi as compared with the mutant viruses, which was at 16 hpi. Reduced titer and slow growth of mutant viruses of FMDV are also reported earlier (Uddowla et al. 2012) and our results support that C-terminal 3A and 3B1 are not essential for virus replication, but can appreciably reduce the rate of production of infectious FMDV progeny particles (Li et al. 2020).

The virus antigens harvested from the deletion mutants and wild-type virus produced a similar antigenic profile of capsid proteins. The TEM images showed the comparable size of virus capsid particles with wild-type virus (Fig. 4c). Interestingly, the dissociation temperature of the deletion mutant virus capsids was lower than that of the wild-type virus capsids as revealed by PaSTRy RNA dye release assay (Fig. 4d). We analyzed the particles at constant ionic buffer at pH 7.4 as the purpose was to understand the impact of length of virus genome on the size and stability of virus capsids. It is hypothesized the genome nucleic acids neutralize or interact with basic regions of the capsid protein to initiate packaging. We observe deletion of 114 bases of 3A and 84 bases of 3B had no effect on the size of the capsid particle, but negatively affected the thermal stability of the capsid particle as reported earlier (Rayaprolu et al. 2017). Perhaps, this could be the reason for the low yield of virus antigen in the deletion mutants (Figure S4) and less number of capsid particles per unit area in the exposed field of TEM image (Fig. 4c).

The deletion mutants, $Asia^{\Delta 3A}$ and $Asia^{\Delta 3A3B1}$ that lack 38 amino acids in the C-terminal of 3A did not react with the 3A peptide hyper-immune serum (raised in-house against FMDV 3A 73 to 153 amino acids). FMDV 3A C-terminal

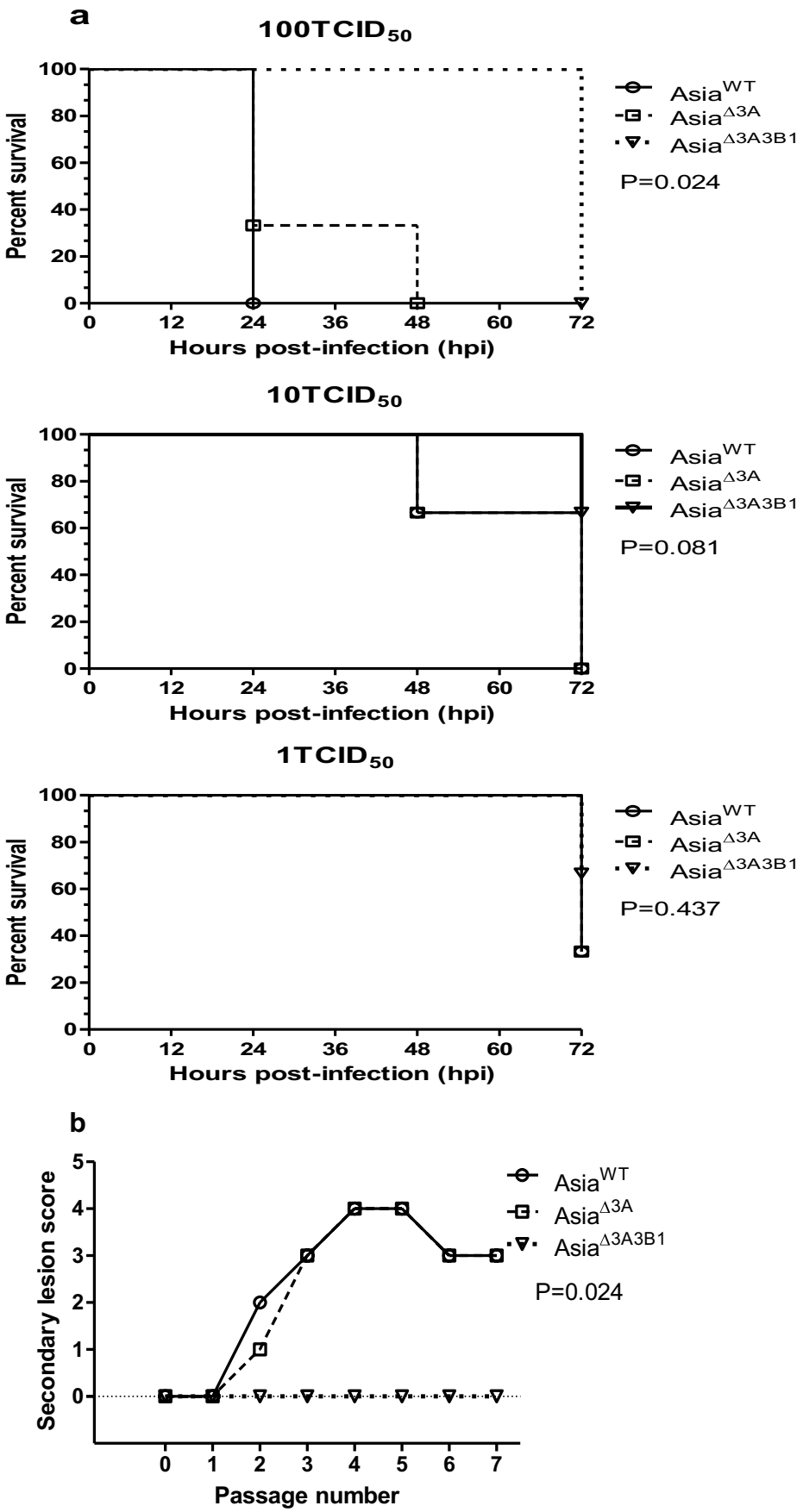

Fig. 5 Experimental infection of foot-and-mouth disease virus (FMDV) Asia1 and its deletion mutants in the laboratory animal models. a Kaplan–Meier curve showing the effect of experimental infection of FMDV Asia1 and its deletion mutants on the survival of suckling mice. Suckling mice ($n=6$/virus type) were infected with 100, 10, and 1 $TCID_{50}$ through the intraperitoneal route (volume 100 µL) and observed for death (endpoint) at 24-h interval for 72 hpi. Log-rank chi-square test for trend revealed a non-significant effect of Asia1 virus types on the survival at 1 and 10 $TCID_{50}$; however, at 100 $TCID_{50,}$ a significant difference in the rate of survival was recorded. At 100 $TCID_{50}$, all the suckling mice in the $Asia^{WT}$ group died at 24 hpi, the $Asia^{\Delta 3A}$ group died at 48 hpi, while complete death was recorded at 72 hpi in the $Asia^{\Delta 3A3B1}$ group ($P=0.024$). At 10 $TCID_{50}$, 67% (4/6) of the mice in the $Asia^{\Delta 3A3B1}$ were alive at 72 hpi. At 1 $TCID_{50}$, 33% of the mice were alive in $Asia^{WT}$ and $Asia^{\Delta 3A}$, while it was 67% in the $Asia^{\Delta 3A3B1}$ group. A mock-infected control group was maintained for each of the experiment. **b** Infectivity of FMDV Asia1 wild-type virus and its deletion mutants in guinea pigs. Adult guinea pigs ($n=4$/group/passage) were infected with 1×10^5 $TCID_{50}$ with $Asia^{WT}$, $Asia^{\Delta 3A}$, and $Asia^{\Delta 3A3B1}$ at the right hind footpad and observed at 24-h interval for 7 days. Appearance of vesicles at the site of inoculation (right hind footpad), defined as a primary lesion, was invariably recorded for the mutant viruses and at every passage. Appearance of vesicles in other footpads was defined as a secondary lesion that was considered infectious in the host. Vesicular fluid and tissue were collected from the affected area between days 3 and 7, and passaged serially in the guinea pigs for seven times. Non-parametric Kruskal–Wallis test with Dunn's post hoc test indicated a significant decrease in the secondary lesion score in the $Asia^{\Delta 3A3B1}$ group as compared with $Asia^{WT}$ virus ($P<0.024$)

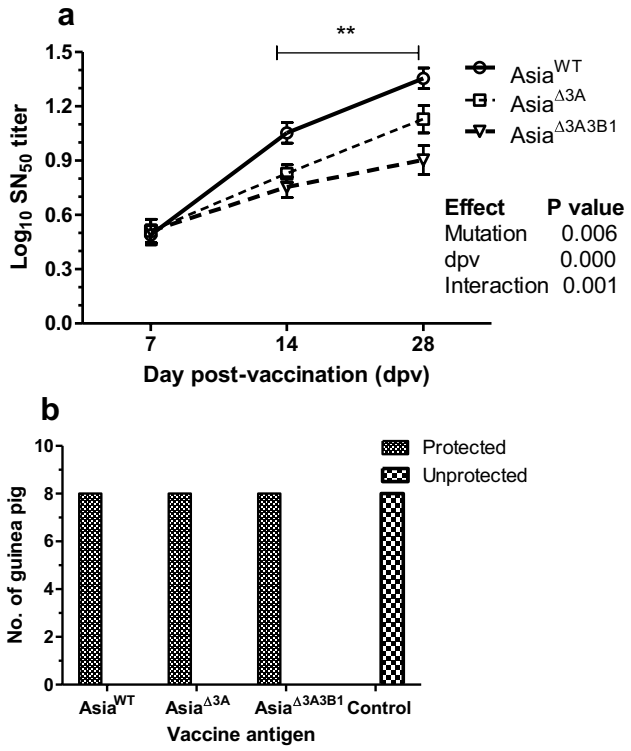

Fig. 6 Immune and protection responses to inactivated antigens of foot-and-mouth disease virus (FMDV) Asia^{WT}, Asia$^{\Delta 3A}$, and Asia$^{\Delta 3A3B1}$ in guinea pigs. Virus-specific vaccine formulations were prepared by inactivating the Asia^{WT}, Asia$^{\Delta 3A}$, and Asia$^{\Delta 3A3B1}$ viruses with binary ethyleneimine and were adjuvanted with montanide ISA 201. Each type of vaccine was administered intramuscularly at the rate of 1 µg/dose in guinea pigs ($n=8$/group). The fourth group served as mock-vaccinated control. All the experimental guinea pigs were challenged with 100TCID$_{50}$ FMDV Asia1/IND/63/1972 on dpv 30 and observed for the development of vesicular lesions on the footpad for the next 7 days. **a** Immune response: Log$_{10}$ SN$_{50}$ titer was determined from the sera collected on days 7, 14, and 28 post-vaccination (dpv). Data was analyzed by two-way repeat measures Anova with Bonferroni post hoc test. Orthogonal contrast was applied to find the pair-wise mean difference with respect to the Asia^{WT} virus as control. Each data point in the line chart indicates mean ± standard error ($n=8$). During the serum neutralization test, each sample was analyzed in duplicate and the average was considered as a data point. A mock-vaccinated negative control was also used (data not shown). Though a significant decrease in the neutralizing antibody titer of Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ viruses was recorded as compared with Asia^{WT} virus on dpv 14 and 28, the guinea pigs were clinically protected ($P<0.05$). **b** Protection response: primary lesion, defined as the appearance of vesicles filled with fluid at the site of challenge, was invariably present following 3–5 days post-challenge. Development of secondary lesions such as vesicles in other footpads was considered a lack of protection response to the vaccine. Protection response was complete (100%) for vaccine antigens of Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ to the challenge virus and was comparable with that of parental wild-type virus

region has dominant immunogenic epitopes, such as 91–104 amino acid (Höhlich et al. 2003), 109–115 amino acid (Fu et al. 2017), and 126–130 amino acid (Wang et al. 2019), which reacted with wild-type virus showing multiple protein bands of the precursor 3AB (Fig. 4b2). It is notable that the mutations did not alter the antigenicity of the capsid protein, which is an essential attribute for a vaccine candidate to elicit the immune response (Fig. 4b1). At the same time, non-reactivity to the targeted gene deletions indicates the potential for DIVA capability. Furthermore, both the mutant viruses were stably maintained up to 20 serial passages in BHK-21cells and also replicated in the suckling mice.

Virulence of the deletion mutants was demonstrated in the suckling mice and guinea pig models. The lethality of Asia$^{\Delta 3A3B1}$ was approximately tenfold less than that of Asia^{WT} in the suckling mice. Though partial deletion of 3A or one copy of 3B did not abolish the growth of virus in the suckling mice, it delayed the death of mice by 48 h as compared to wild-type and Asia$^{\Delta 3A}$ virus. Furthermore, Asia$^{\Delta 3A3B1}$ failed to produce clinical lesions up to seven serial blind passages following footpad infection in guinea pigs. Milder primary lesions at the site of infection indicated that the deletion of a region of FMDV 3A and a copy of 3B together had a negative pathogenic effect on the virus replication in guinea pigs. Similarly, the infectious viral progenies were recovered from the BHK21 cells using tissue samples collected from experimentally infected mice. However, in guinea pigs, Asia$^{\Delta 3A3B1}$ could not be recovered. Furthermore, the Asia$^{\Delta 3A3B1}$ virus did not induce FMDV-specific antibody on dpi 30 in guinea pigs indicating the absence of virus replication (Figure S5). Thus, FMDV has considerable flexibility with respect to 3A and 3B both of which influence the pathogenic potential of the virus and host range (Pacheco et al. 2013). Deletions in 3A and deletion of redundant copies of 3B are associated with attenuation of the virus in the cattle (Pacheco et al. 2013) but with high virulence in swine (Núñez et al. 2007). Deletion of 3B did not significantly attenuate the virus *per se* due to the presence of leader sequence, which is shown to be responsible for virus virulence (Uddowla et al. 2012). In this study, guinea pigs produced disease only when infected with Asia$^{\Delta 3A}$ virus but not with Asia$^{\Delta 3A3B1}$ virus infection, which lacked 3A and 3B together.

Furthermore, administration of inactivated vaccine prepared from the deletion mutants protected the guinea pigs when challenged with the homologous virulent virus on dpv 30, though the serum neutralizing antibody was significantly low for Asia$^{\Delta 3A}$ and Asia$^{\Delta 3A3B1}$ as compared with the wild-type Asia. However, the precise duration of protection with or without booster administration needs to be determined as antibody titer will decline over a period of time. Current commercial trivalent FMDV grown in large manufacturing units has the risk of escape of virulent virus. Furthermore, the removal of contaminated NSP increases the cost of vaccine production. In this context, the double gene-deleted negative marker virus vaccine

using Asia$^{\Delta 3A3B1}$ is safe as far as virulence is concerned and the multiple deletions pose the least possibility of reversion to wild-type virus. Furthermore, in an emergency outbreak situation that demands vaccine strain, replacement is facilitated by swapping the structural protein P1 region with flanking primers, which keeps the negative marker region intact for DIVA capability (Biswal et al. 2015b). In an outbreak scenario, the diagnosis of infected animals can be double confirmed by PCR of the genetic material of the virus for the deleted region and in conjunction with companion DIVA ELISA. The new vaccine platform opens the possibility of a "vaccinate-to-live" policy in FMD-free countries in emergency outbreak conditions. Studies on the infectivity, vaccine efficacy, and DIVA compatibility of the Asia$^{\Delta 3A3B1}$ double gene-deleted negative marker virus vaccine need to be demonstrated in the natural hosts.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-021-11692-2.

Acknowledgements The authors thank the Director, ICAR-IVRI, Izatnagar and Joint Director, ICAR-IVRI, Bengaluru, for facilitating the research work. The authors also acknowledge SEPPIC-Air Liquide Healthcare Specialty Ingredients for providing Montanide ISA-201 VG adjuvant.

Author contribution H.L.: part of the PhD work, production of deletion clones, and characterization; S.E. and J.K.B.: cell culture and rescue of the virus. Initial infectious cDNA clone modification; R.P.T.S. and A.S.**:** serotyping differentiating ELISA; LPBE; N.K., G.R.R., and V.B.: statistical analysis, presentation of the graph, and TEM; P.S., M.P., and V.P.: animal experimentation and qPCR; H.J.D.: conception of the idea, overall execution, and manuscript preparation. All authors read and approved the manuscript.

Funding The work was supported by CAAST-ACLH (NAHEP/CAAST/2018–19) of ICAR-World Bank funded National Agricultural Higher Education Project (NAHEP). The first author was a recipient of UGC-Maulana Azad National Fellowship (Ref No.F117.1/201415/MANF201415CHRMIZ36717).

Data Availability The data will be made available upon reasonable request.

Code availability Not applicable.

Declarations

Ethics approval The animal experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) *vide* Letter F. No.8–56/Vol II/RCSS/2018–19/2 and carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA), Government of India.

Consent to participate We agree to participate.

Consent for publication We agree to publish in Applied Microbiology and Biotechnology.

Conflict of interest The authors declare no competing interests.

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