Applied Microbiology and Biotechnology (2023) 107:1285–1297
https://doi.org/10.1007/s00253-023-12359-w

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Thermostable negative-marker foot-and-mouth disease virus serotype O induces protective immunity in guinea pigs

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Received: 11 August 2022 / Revised: 17 December 2022 / Accepted: 31 December 2022 / Published online: 19 January 2023

Abstract

Foot-and-mouth disease (FMD) is a contagious viral disease of high economic importance, caused by FMD virus (FMDV), a positive-sense single-stranded RNA virus, affecting cloven-hoofed animals. Preventive vaccination using inactivated virus is in practice to control the disease in many endemic countries. While the vaccination induces antibodies mainly to structural proteins, the presence of antibodies to the non-structural proteins (NSP) is suggestive of infection, a criterion for differentiation of infected from vaccinated animals (DIVA). Also, there is a growing demand for enhancing the stability of the FMD vaccine virus capsid antigen as the strength of the immune response is proportional to the amount of intact 146S particles in the vaccine. Considering the need for a DIVA compliant stable vaccine, here we report generation and rescue of a thermostable and negative marker virus FMDV serotype O (IND/R2/1975) containing a partial deletion in non-structural protein 3A, generated by reverse genetics approach. Immunization of guinea pigs with the inactivated thermostable-negative marker virus antigen induced 91% protective immune response. Additionally, a companion competitive ELISA (cELISA) targeting the deleted 3A region was developed, which showed 92.3% sensitivity and 97% specificity, at cut-off value of 36% percent inhibition. The novel thermostable-negative marker FMDV serotype O vaccine strain and the companion cELISA could be useful in FMDV serotype O enzootic countries to benefit the FMD control program.

Key points

- *Thermostable foot-and-mouth disease virus serotype O with partial deletion in 3A.*
- *Inactivated thermostable marker vaccine induced 91% protection in guinea pigs.*
- *Companion cELISA based on deleted region in 3A could potentially facilitate DIVA.*

Keywords Foot-and-mouth disease · Differentiation of infected from vaccinated animals · Thermostable negative-marker · Vaccine · Competitive ELISA

Introduction

Foot-and-mouth disease (FMD) is a contagious and economically important viral disease of cloven-hoofed animals including cattle, buffalo, pigs, sheep, and goats. The disease is reported to circulate in livestock population in Africa, the Middle East, and Asia, as well as in parts of South America. Estimated disease burden is highest in China, India, the Near East, and the Sahel (North Africa) (Sumption et al. 2008). The disease causes negative economic impact on animal health, productivity, and international trade (Subramaniam et al. 2013). The annual losses affecting production and vaccination costs in endemic regions amount to US$ 6.5–21 billion (Knight-Jones and Rushton 2013). The causative FMD virus (FMDV) with positive sense ssRNA (8.5 kilo nucleotides) genome belongs to the genus *Aphthovirus* of family *Picornaviridae* (Knowles and Samuel 2003). The virus comprises of four structural proteins (VP1-4) and several non-structural proteins (L, 2A, 2B, 2C, 3A, 3B1-3, 3C, and

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3D). There are seven serotypes of FMDV which include serotype A, O, Asia1, C, SAT1, SAT2, and SAT3, with several subtypes (Bachrach 1968). Serotype O is the most prevalent in India, causing ~ 98% FMD outbreaks (Singh et al. 2019).

The control of FMD in endemic region is predominantly by vaccination with chemically inactivated virus vaccine. One of the limitations with FMD vaccine antigen is dissociation of intact virions (146S) into less immunogenic pentameric subunits (12S) upon exposure to moderate heat. FMDV serotype O capsid is relatively more sensitive to heat and acidic pH (Doel and Baccarini 1981). This necessitates maintaining cold chain for the storage and delivery of the vaccine to preserve its antigenicity. It was previously shown that the amino acid substitution in VP2 (Y2098F) produced thermostable capsid (Ganji et al. 2018; Gao et al. 2021; Kotecha et al. 2015; Scott et al. 2017). We recently demonstrated that cattle vaccinated with inactivated thermostable virus with Y2098F produced higher neutralizing antibody titer compared to the parental O IND/R2/1975 vaccine (Biswal et al. 2022). Moreover, for effective implementation of disease control program, in long-term eradication campaigns, it is essential to have marker vaccines which allow for unequivocal distinction between infected and vaccinated animals during post-outbreak serological surveys (Mason and Grubman 2009). Apparently, the current inactivated vaccines are not completely NSP free, and antibody response is elicited against them with the repeat immunizations (Lee et al. 2006), thus hampering precise sero-surveillance. Previously, negative marker vaccines were developed targeting some of the structural and/or non-structural proteins in FMDV. The deletion of G–H loop in VP1 (Fowler et al. 2014), leader coding region (Lpro), and 3B1/3D proteins have been evaluated for DIVA compliance (Uddowla et al. 2012). It was shown that carboxyl-terminal of 3A region, 3B1, and 3B2 can accommodate deletions without compromising the replication efficiency of the virus (Pacheco et al. 2003; Li et al. 2014; Biswal et al. 2015; Behura et al. 2016; Bhatt et al. 2018; Dhanesh et al. 2020; Lalzampuia et al. 2022). In this study, with the aim of producing FMDV vaccine with negative marker attribute and containing thermostable capsid, we generated a deletion mutant in 3A C-terminal region of O IND/R2/1975 virus with thermostable capsid having Y2098F mutation. A competitive ELISA was also developed in this study as a companion test for the negative-marker vaccine. The newly generated candidate thermostable-negative marker virus vaccine induced enhanced immune response in guinea pigs apart from facilitating DIVA compliance, highlighting its relevance during eradication stage of the disease, potentially to distinguish vaccinated animals from inapparently infected carriers in a herd.

Materials and methods

Cell culture and virus

Baby hamster kidney 21 (BHK-21) (clone 13; ATCC, USA, #CCL-10) cell line was maintained in Glasgow Modified Essential Medium (GMEM) (HiMedia, India, #AT1010) supplemented with 10% fetal bovine serum (HiMedia, India, #RM10681) and 60 µg/ml penicillin (Sigma, USA, #P3032), 100 µg/ml streptomycin (Sigma, USA, #S9137), and 100 µg/ml kanamycin (Sigma, USA, #K1377) at 37 °C in 5% CO_2. The Indian vaccine strain FMDV O/IND/R2/1975 (monolayer passage #5) maintained at the FMD vaccine laboratory, IVRI, Hebbal, Bengaluru, was used for the generation of infectious cDNA clone.

Experimental animals

New Zealand white rabbits were used to produce polyclonal antibodies. Dunkin Hartley guinea pigs were used for the vaccine efficacy testing. The animal experimental protocols were carried out in compliance with the guidelines of the Institute Animal Ethics Committee of ICAR-Indian Veterinary Research Institute regional campus, Bengaluru (Approval Nos. IVRI BNG/2019–20/79 and IVRI BNG/2019–20/81).

Generation of recombinant cDNA clone

Four contiguous fragments T7-SF, LF-VP4, VP4-3A, and 3A-Poly(A) covering the entire FMDV genome were PCR amplified with KOD hot start DNA polymerase (Merck, USA, #71,086), using cDNA prepared from RNA isolated from FMDV serotype O/IND/R2/1975. The primers used for the amplification are given in Table 1 (Eurofins Genomics, India, # EGI-Oligos-002).

In vitro sequential ligation-based approach was followed to clone the full-length viral sequence downstream of the T7 promoter into the pT7 blue blunt vector (Merck, USA, #70,174). The recombinant plasmid was designated as pT7-OR2/75. The viral VP2 region constituting thermostable mutation Y2098F was amplified from thermostable virus (Biswal et al. 2022) using the L482F and EcoRI-R3 primers. The amplicon was digested with *Nhe* I (New England Biolabs, USA, #R0131S) and *Bsu*36 I (New England Biolabs, USA, #R0524S) and used to replace the corresponding fragment in pT7-OR2/75. The new recombinant plasmid was designated as pT7-OY2098F. Partial deletion of 87 to 144 amino acid residues in non-structural protein 3A region was carried out using inverse PCR (Erster and Liscovitch 2010) in pT7-OY2098F plasmid, using primers

Table 1 Sequences of primers used in the study

Primer	Nucleotide sequence* (5′-3′)
EcoRV-T7-F1	AT**GATATC**TAATACGACTCACTATAGGGTTTGAAAGGGGGC
SmaI-R1	TAAT**CCCGGG**GGGGGGGGGGGGGGGGATGAAAGACGGGC
SmaI-F2	TAT**CCCGGG**CCCCCCCCCCCCCCCCCTAAGTTTTACCGTCGTTC
NheI-R2	CTG**GCTAGC**TTTGAAAACCAGTCGTTG
NheI-F3	GTTTTCAAA**GCTAGC**CAGCTCTGCTTTTAG
EcoRI-R3	TCAAA**GAATTC**AATTGCAGCCTCGTGTTG
EcoRI-F4	ATT**GAATTC**TTTGAGGGAATGGTGC
StuI-R4	CG**AGGCCT**TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
Δ3A-144-F	CCCGTGGAGGAACAACCACAAGCTG
Δ3A-87-R	CACCATCTGCTGTCTCTTGCGAGTC
DHP13	GTGACTGAACTGCTTTACCGCAT
DHP15	CAACGGGACGARCAAGTACTC
DHP9	GACCTGGAGGTYGCGCTTGT
NK61	GACATGTCCTCCTGCATCTG
L482F	ACCTCCRACGGGTGGTACGC

*Restriction enzyme sites are indicated in bold

Δ3A-144-F and Δ3A-87-R. The resulting recombinant plasmid was designated as pT7-OY2098FΔ3A. Since the deletion region of 3A was flanked by *Eco*RI site on both sides, the *Eco*RI fragment from pT7-OY2098FΔ3A was used to replace the corresponding fragment in pT7-OR2/75, generating a plasmid designated as pT7-OR2/75Δ3A. The deleted region encompassed three linear FMDV-specific B-cell epitopes (Hohlich et al. 2003). All the recombinant plasmids were confirmed by restriction enzyme analysis and automated DNA sequencing.

In vitro transcription and rescue of recombinant FMD viruses

The plasmids pT7-OR2/75, pT7-OY2098F, pT7-OR2/75Δ3A, and pT7-OY2098FΔ3A were linearized with *Stu* I restriction enzyme (Thermo Fisher Scientific, USA, #FD0424) and in vitro transcribed using T7 High Yield RNA Synthesis kit (New England Biolabs, USA, #E2040S). RNA transfection was performed using Lipofectamine 3000 reagent (Thermo Fisher Scientific, USA, #L3000008) into 1×10^6 BHK-21 cells and incubated at 37 °C with 5% CO_2. Virus in cell culture supernatant was used for the blind passage in BHK-21 cells until typical cytopathic effect (CPE) was observed. The wild-type (WT) and thermostable (Y2098F) viruses as well as their respective deletion mutants WTΔ3A and Y2098FΔ3A were rescued. The rescued viruses were confirmed by nucleotide sequencing following 12 passages.

Serotype identity and antigenic relationship

The serotype identity of the rescued viruses was confirmed by using FMDV serotype-specific multiplex PCR by using a combination of serotype-specific primers—DHP13, DHP15, DHP9, and NK61 (Giridharan et al. 2005). For antigenic confirmation, sandwich ELISA (Bhattacharya et al. 1996) was performed by using serotype-specific rabbit serum raised against the FMDV 146S antigen as capture antibody and hyperimmune serotype-specific guinea pig serum raised against the FMDV 146S antigen as tracer antibody. The antigen complex was detected by horseradish peroxidase (HRP)–conjugated rabbit anti-guinea pig immunoglobulin (Dako, Denmark, # P0141). Further, the antigenic relationship of recombinant viruses in comparison with parent wild-type virus was assessed using a two-dimensional micro-neutralization test, by determining r1-value as described previously (Dhanesh et al. 2020). Briefly, the r1-value of recombinant viruses in relation with the currently used vaccine strain O/IND-R2/1975 was calculated by using bovine vaccinate serum (BVS) raised against serotype O FMDV (Rweyemamu et al. 1978). The titer of each virus and titer of the serum at each dilution of individual viruses were determined by the endpoint dilution method. Further, the serum neutralization titer at 100 $TCID_{50}$ of each virus was determined, and the antigenic coverage of the rescued viruses is expressed as r1-value, calculated as below:

$$r1 = \frac{Titer\ of\ rescued\ virus\ against\ BVS}{Titer\ of\ wildtype\ O\ IND/R2/1975\ against\ BVS}$$

Plaque morphology assay

Plaque assay was performed as described previously (Bachrach et al. 1957). Briefly, BHK-21 monolayer cells were infected with 500 µl of 10^{-5} diluted viral samples, incubated at 37 °C for 1 hour (h). The cells were then washed and overlaid with 1.5% Sea Plaque® agarose (Lonza, Switzerland, #50101) and incubated at 37 °C for 48 h. The plaque morphology was observed after fixing with 10% formaldehyde and staining with 0.1% crystal violet (Sigma, USA, #C3886).

Multistep growth curve analysis

The growth kinetics of rescued viruses was analyzed by infecting BHK-21 cells at a multiplicity of infection (MOI) of 0.01. After 1 h adsorption, the cells were washed twice with GMEM (pH 6.0) and supplemented with complete medium. Supernatant was harvested at 0, 1, 2, 4, 6, 8, 10, 12, 18, and 24 hour post-infection (hpi). The virus titer at each time point was estimated by endpoint dilution method (Reed and Muench 1938).

Thermal inactivation assay

The thermal stability of the rescued Y2098FΔ3A virus in comparison to wild-type and WTΔ3A viruses was assessed by incubating the viruses at 42 °C for 30, 60, 90, 120, 180, and 240 min, and at 49 °C for 15, 30, 45, and 60 min, followed by infection in BHK-21 cells. The virus titer was determined by endpoint dilution method (Reed and Muench 1938).

Immunofluorescence assay

BHK-21 cell monolayer was infected with recombinant FMDV mutant viruses at 0.1 MOI for 8 h, in 24-well dish, fixed using 4% paraformaldehyde (HiMedia, India, #TCL119) for 15 min at 4 °C and permeabilized with 0.1% Triton-X100 (Amresco, USA, #0694) in Tris-buffered saline (TBS) for 15 min. The cells were blocked with TBS with 3% BSA (Alfa Aesar, USA, #J64655) for 30 min and then incubated with 1: 100 diluted 3B-specific mAb, 10H9D8 (Hosamani et al., 2022), at 37 °C for 1.5 h. After each step, washing was carried out with TBS containing Tween 20 (0.05%). The cells were then probed with Alexa Fluor 488 goat anti-mouse IgG antibody (1:500) (Thermo Fisher Scientific, USA, #A-11001) for 1 h and visualized under fluorescence microscope (Nikon eclipse Ti-S).

Expression and purification of t3A (3A 87–144) protein

The plasmid pET32a t3A 87–144 containing deletion region (87–144 amino acids) of 3A protein of FMDV A-IND40/00 in the modified pET-32a(+) vector (Merck, USA, #69015) without thioredoxin tag was prepared previously (Dhanesh et al. 2020). The plasmid was transformed into BL21(DE3)pLysS competent *E. coli* cells (Merck, USA, #69451), and positive colonies were selected by using chloramphenicol (34 µg/mL) and ampicillin (50 µg/mL). The recombinant *E. coli* culture was induced by 1.5 mM IPTG for 6 h, the cell pellet was lysed, and the Ni–NTA agarose (Qiagen, Germany, # 30210) affinity chromatography was used to purify the expressed protein. The elution was made with 300 mM imidazole in the elution buffer. The fractions were pooled and dialyzed overnight against Tris-NaCl buffer (pH: 8.0) in Slide-A-Lyzer® Dialysis cassette (Thermo Fisher Scientific, USA, #66810). The dialyzed protein was concentrated in Pierce® Concentrator 3 K MWCO (Thermo Fisher Scientific, USA, # 88525).

Raising hyperimmune serum

The monospecific polyclonal serum was raised by immunizing rabbit with *E. coli* produced t3A polypeptide and boosting subsequently on days 21 and 42 post first immunization, with KLH-conjugated synthetic peptide TTDDKTLDEAEKNPL ('S' Biochem, India, #Pep-255), a linear B-cell epitope in the deleted region of the 3A protein (Fu et al. 2017; Hohlich et al. 2003).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

SDS-PAGE and western blotting of virus-infected BHK-21 cell lysate was carried out as described earlier (Ranjitha et al. 2020). Briefly, the protein-blotted PVDF membrane (Merck, USA, #GE10600089) was probed using rabbit monospecific serum raised against t3A, 3D-specific mAb (6B8D11) (Kushwaha et al. 2021), 3B-specific mAb (10H9D8), rabbit serum against serotype O capsid antigen or GAPDH (Santa Cruz, USA, #SC-59540) (dilution 1:500 for all), followed by species-specific HRP-conjugated secondary antibody (1:1000) (Dako, Denmark, #P0448 [anti-rabbit] and #P0260 [anti-mouse]), and membrane visualization using Clarity Western ECL substrate (Bio-Rad, USA, #1705060) in a chemiluminescence imager (Uvitec, UK).

Thermofluor PaSTRy assay of recombinant viruses

The assay was performed as described previously (Kotecha et al. 2016) using AB 7500 real-time PCR machine (Applied Biosystems, USA) with SYBR green II dye (Thermo Fisher Scientific, USA, #S7564). Briefly, purified and concentrated

viruses were prepared by 25% sucrose cushioning ultracentrifugation, followed by CsCl gradient ultracentrifugation. For the PaSTRy assay, 20 µl volume reactions with 0.1–0.5 µg viruses were set up in duplicate along with SYBR green II dye at 5 × final concentration. The samples were subjected to a recurring increase in temperature from 25 to 95 °C with 1% ramping. The liberation of RNA from virus capsids was tracked by an increase in fluorescence intensity, and the melting temperature was recorded.

Antigen production and vaccine formulation

Bulk production of antigen was done by infecting monolayer of BHK-21 cells with the viruses in roller bottles. Three mM binary ethyleneimine (BEI) prepared in 0.7% NaOH was used for the inactivation of the virus for 24 h at room temperature. Subsequently, the inactivated virus was concentrated with 8% PEG-6000 and quantified using spectrophotometer, following purification of the 146S fractions by cesium chloride density gradient ultracentrifugation. The separate vaccine preparation of each virus in 250 µl dose containing 2 µg of PEG concentrated inactivated antigen was formulated in double oil emulsion using Montanide ISA 201 VG adjuvant (Seppic, France) at 1:1 w/w ratio.

Vaccination and potency testing in guinea pigs

Three groups of guinea pigs ($n = 12$ per group) were injected intramuscularly on day 0 and boosted on the 28th day post-vaccination (DPV) with vaccine prepared using Y2098FΔ3A, WTΔ3A, or wild-type virus antigens. The control group was injected with phosphate-buffered saline. Blood samples were collected on the 28th DPV and 56th DPV. On 56th DPV, all the groups were challenged with 50 µl volume containing 100 $GPID_{50}$ of guinea pig–adapted wild-type FMDV O IND/R2/1975 by footpad injection. The animals were examined daily for 8 days for the development of primary and secondary lesions. Blood serum was collected from virus-challenged animals on the 14th day post-challenge (DPC).

Indirect enzyme-linked immunosorbent assay (ELISA)

Vaccinated serum samples collected from guinea pigs on the 28th DPV, 56th DPV, and 14th DPC were assayed by indirect ELISA test performed using t3A antigen-coated plate. Briefly, Nunc MaxiSorp plates (Thermo Fisher Scientific, USA, #44–2404-21) were coated with t3A antigen (50 ng/well) in carbonate-bicarbonate buffer (Sigma, USA, #C3041) by incubating at 4 °C overnight. After blocking for 60 min at 37 °C with blocking buffer 3% SMP in phosphate-buffered saline containing Tween 20 (0.05%) (PBS-T), the plates were incubated with guinea pig serum (collected on the 28th DPV, 56th DPV, and 14th DPC) in blocking buffer (1:20 dilution), for 1 h at 37 °C, and then probed with anti-guinea pig HRPO conjugate (Dako, Denmark, # P0141) at 1:3000 dilution for 1 h at 37 °C. Following each step, the plates were washed thrice with PBS-T. The reaction was developed by incubation with hydrogen peroxide (Merck, USA, #108597) and o-phenylenediamine (Sigma, USA, #P9029) for 15 min at 37 °C. Absorbance was read at 492 nm using an ELISA plate reader (Tecan, Switzerland).

Virus neutralization test (VNT)

Virus neutralizing antibodies in the sera of vaccinated guinea pigs were determined following the procedure described previously (WOAH 2022) using wild-type FMDV serotype 'O' virus. In brief, the serum was serially two-fold diluted from 1:8 to 1:1024 and incubated with 100 $TCID_{50}$ of wild-type FMD virus for 1 h at 37 °C and, after that, 50,000 BHK-21 cells added per well. The plates were incubated for 48 h at 37 °C and observed for the CPE to calculate the VN_{50} titer. The experiment was performed in duplicate, and the mean $Log_{10}VN_{50} \pm SD$ of each experimental group was determined.

Competitive enzyme-linked immunosorbent assay (cELISA)

Nunc MaxiSorp plates (Thermo Fisher Scientific, USA, #44–2404-21) were coated with recombinant antigen t3A (50 ng/well). A panel of 211 bovine sera samples available in our laboratory, consisting of naïve unvaccinated FMD seronegative ($n=46$), FMD-vaccinated ($n=87$), and FMDV-infected ($n=78$) cattle, was used in the assay. The rabbit monospecific polyclonal serum was used as detection antibody. Known FMDV-infected bovine sera and known negative bovine serum were used as positive and negative control, respectively. The test cattle serum was premixed with equal volume of antigen-specific rabbit monospecific polyclonal serum (final dilution 1:7000) and added to antigen-coated plate for incubation 37 °C for 1 h. Then, anti-rabbit IgG horseradish peroxidase (HRP) conjugate at dilution of 1:3000 (Dako, Denmark, #P0448) was used as secondary antibody for 1 h at 37 °C. Following each step, the plates were washed thrice with PBS-T. The reaction was developed by incubation with hydrogen peroxide (Merck, USA, # 108597) and o-phenylenediamine (Sigma, USA, #P9029) for 15 min at 37 °C. Absorbance was read at 492 nm using an ELISA plate reader (Tecan, Austria). MedCalc software was used to determine the cut-off based on the percent inhibition (PI) values obtained for each sample. Receiver operating characteristic curve (ROC) was analyzed as described previously (DeLong et al. 1988). Diagnostic sensitivity and specificity were compared with that of Prio-CHECK FMDV NS ELISA (Thermo Fisher Scientific, USA, #7610440).

Statistical analysis

Statistical analyses were performed using GraphPad Prism v9. The Student's *t*-test was performed to identify statistical significance, and *p*-value of less than 0.05 was considered significant.

Results

Serotype confirmation and antigenic relationship of the rescued recombinant viruses

Multiplex PCR was performed for the serotype confirmation of the rescued viruses by using the combination of primers—DHP13, DHP15, DHP9, and NK61. Multiplex PCR showed a single band of 249 bp as expected for serotype O (Fig. 1A). In addition, serotype specificity of rescued viruses was confirmed by sandwich ELISA (Fig. 1B). The estimated r1-value was 0.98 and 1.0 for WTΔ3A and Y2098FΔ3A, respectively, when compared with wild-type vaccine strain O IND/R2/1975 (Figure S1).

Genetic stability of the thermostable mutation

No reversion was observed in the VP2 mutation site nor the 3A deletion site of the viruses, after 12 passages in BHK-21 cells (Fig. 2A and B). The titer of the rescued recombinant viruses increased by sequential propagation in BHK-21 for 10–12 passages. The estimated mean titer (log_{10} $TCID_{50}$/mL) of WT, WTΔ3A, and Y2098FΔ3A in BHK-21 cells was 7.9, 7.9, and 7.4, respectively (Fig. 2C).

Antigenic reactivity and profiling of the rescued negative marker thermostable virus

The antigenic reactivity of rescued FMD viruses was assayed by immunofluorescence assay. BHK-21 cells were mock infected or infected with rescued viruses and probed with

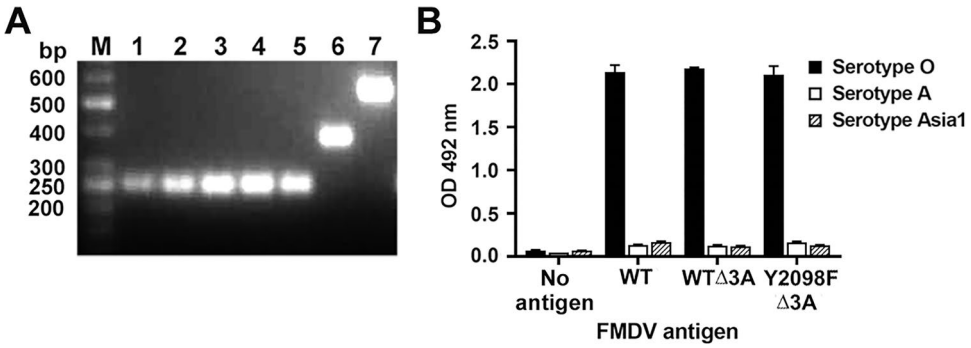

Fig. 1 Identification of the serotype of rescued viruses. **A** Gel electrophoresis of the multiplex PCR assay for the identification of serotype of the recombinant viruses. Lane M: 100 bp DNA ladder, Lanes 1–4, amplicon of 249 bp of WT, WTΔ3A, Y2098F, and Y2098FΔ3A of the rescued viruses, respectively. Lanes 5, 6, and 7: positive control amplicons from serotypes O (249 bp), A (376 bp), and Asia 1 (537 bp) respectively. **B** Serotype differentiation sandwich ELISA of the rescued viruses—WT, WTΔ3A, and Y2098FΔ3A showing reactivity with serotype O-specific serum

Fig. 2 Confirmation of the genetic stability of the rescued recombinant viruses and their characterization. **A** Multiple sequence alignment of the VP2 region of WT, WTΔ3A, Y2098F, and Y2098FΔ3A after 12 passages in BHK-21 cells. The presence of phenylalanine (F) at 98th position in place of tyrosine (Y) confirms the presence of mutation in Y2098F and Y2098FΔ3A. **B** Multiple sequence alignment of the 3A non-structural protein region of WT, WTΔ3A, Y2098F, and Y2098FΔ3A after 12 passages in BHK-21 cells. The presence of deletion at 87–144 amino acids in WTΔ3A and Y2098FΔ3A confirms the deletion. **C** The titers of the rescued viruses—WT, WTΔ3A, and Y2098FΔ3A in the tissue culture supernatants, determined by $TCID_{50}$ analysis and mean titer expressed as $Log_{10}TCID_{50}$/ml

3B-specific mAb (10H9D8). The cells infected with WT as well as rescued negative marker viruses showed detectable fluorescence, suggesting virus replication. This was absent in the mock-infected control cells (Fig. 3A).

The antigenic profiling of the rescued viruses using rabbit monospecific polyclonal serum against deleted 3A region showed similar bands in the range of 16–32 kDa in wild type and Y2098F, but not in the corresponding deletion viruses in WTΔ3A and Y2098FΔ3A. 3B-specific mAb (10H9D8) against 3B precursors detected 24–32 kDa bands in wild-type and Y2098F viruses, while the deletion of 58 amino acids in WTΔ3A and Y2098FΔ3A viruses resulted in 12–20 kDa bands. However, 6B8D11 mAb specific to 3D protein and rabbit serum against FMDV 146S of serotype O detected similar size bands for the non-structural protein 3D (53 kDa) and structural proteins VP3/VP1 (27/25 kDa), respectively, in all four viruses. Control GAPDH was detected in all including the mock-infected cell lysates (Fig. 3B). Original images of western blots are provided in Figure S2.

Plaque morphology and replication kinetics of rescued viruses

In plaque morphology assay utilizing BHK-21 cell monolayers, it was observed that wild-type and Y2098FΔ3A viruses produced medium- to large-sized similar plaques, whereas WTΔ3A produced small-sized plaques on BHK-21 cell monolayers (Fig. 4A). However, the titer of all viruses was similar. The effect of mutation and deletion on the replication kinetics of the viruses was investigated by multistep growth curves analysis. The rescued viruses exhibited analogous replication kinetics. The wild-type, WTΔ3A, and Y2098FΔ3A viruses upon inoculation with 0.01 MOI in a BHK-21 cell monolayer similarly attained maximum titers by 12–18 h post-infection (Fig. 4B).

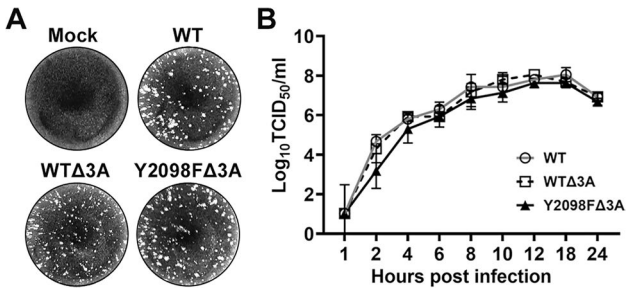

Fig. 4 Growth kinetics of the recombinant viruses. **A** Plaque morphology of wild-type (WT), WTΔ3A, and Y2098FΔ3A recombinant viruses in BHK-21 cell monolayers. **B** Multistep growth kinetic study performed by infecting BHK-21 cells at 0.01 MOI with wild-type (WT), WTΔ3A, and Y2098FΔ3A viruses and titers in tissue culture supernatants determined by $TCID_{50}$ analysis. The average titers with standard deviations expressed as $Log_{10}TCID_{50}$/ml at indicated time points

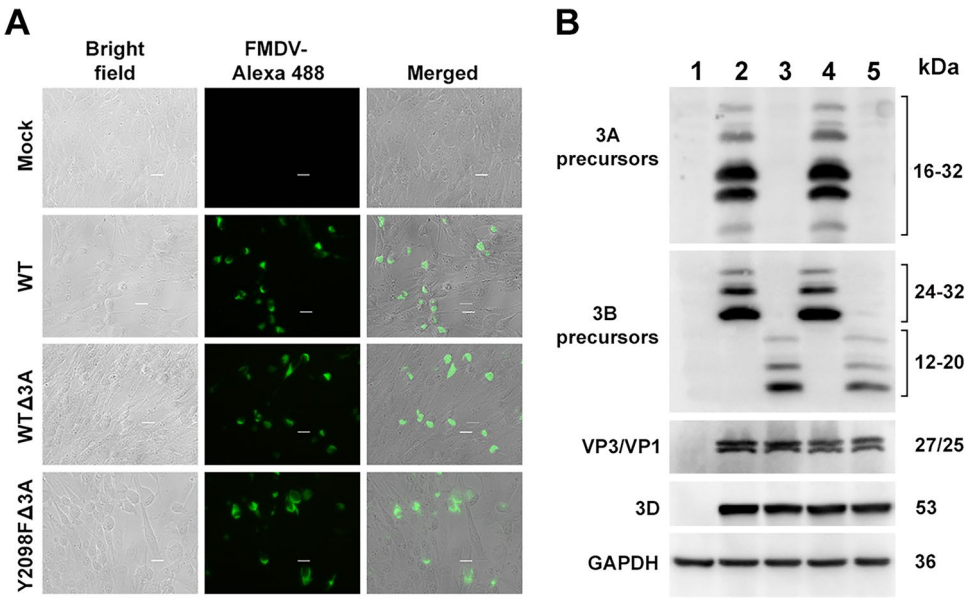

Fig. 3 Antigenic characterization of the recombinant viruses. **A** Bright field and immunofluorescence microscopy images (400× magnification) of BHK-21 cells infected with either WT or rescued negative marker viruses (WTΔ3A and Y2098FΔ3A) showing detectable fluorescence upon probing with 3B-specific mAb, suggesting virus replication. The fluorescence was absent in the mock-infected control cells. Scale bar: 20 µm. **B** Western blot analysis of lysate from the WT or recombinant virus-infected BHK-21 cells, probed with rabbit polyclonal serum against deleted 3A region, 3B-specific mAb (10H9D8), rabbit sera raised against 146S of FMDV serotype O, and 3D-specific mAb (6B8D11). Lane 1, mock infected; lane 2, WT; lane 3, WTΔ3A; lane 4, Y2098F; lane 5, Y2098FΔ3A

Rescued thermostable virus is less liable to thermal dissociation

The capsid thermal stability of recombinant viruses was assessed by ThermoPaSTRy. While the dissociation temperature of wild-type and WTΔ3A viruses was 55.62 °C and 55.43 °C, respectively, Y2098FΔ3A dissociated at 57.91 °C, an increase by 2.48 °C (Fig. 5A).

The stability of the rescued viruses was also studied in comparison with wild-type FMDV by thermo-inactivation assay in BHK-21 cells. The viruses with an approximate titer of $10^{7.4}$–$10^{7.8}$ were incubated at 45 °C and 49 °C for different time points, and residual virus titer was determined. At 45 °C by 4 h, there was a decrease in virus titer of WT, WTΔ3A, and OY98FΔ3A by $\log_{10}$ 2.1, 2, and 0.91, respectively (Fig. 5B). At 49 °C by 1 h, titer of WT, WTΔ3A, and OY98FΔ3A decreased by $\log_{10}$ 2.1, 2.0, and 0.66, respectively (Fig. 5C).

Deletion mutant virus vaccines induced neutralizing antibody response similar to wild-type vaccine in guinea pigs

The neutralizing antibody titer ($Log_{10}VN_{50}$) on the 28th day post-vaccination in guinea pigs was 0.9, 1.0, and 0.8, respectively, for WT, WTΔ3A, and OY98FΔ3A. However, upon boosting on the 28th DPV, the VN_{50} titer on the 56th DPV increased to 1.7, 1.8, and 1.59, respectively. This increase in neutralizing antibody response was significant compared to the 28th DPV response ($p < 0.01$). However, there was no significant difference in the immune response among different vaccinated groups compared to wild-type vaccinated animals (Fig. 6A). Upon challenge with 100 $GPID_{50}$ homologous wild-type virus, control unvaccinated animals showed footpad lesions appearing by the 4th DPC (Figure S3). Whereas WT and WTΔ3A antigen immunized animals showed 75% protection with no footpad lesions in

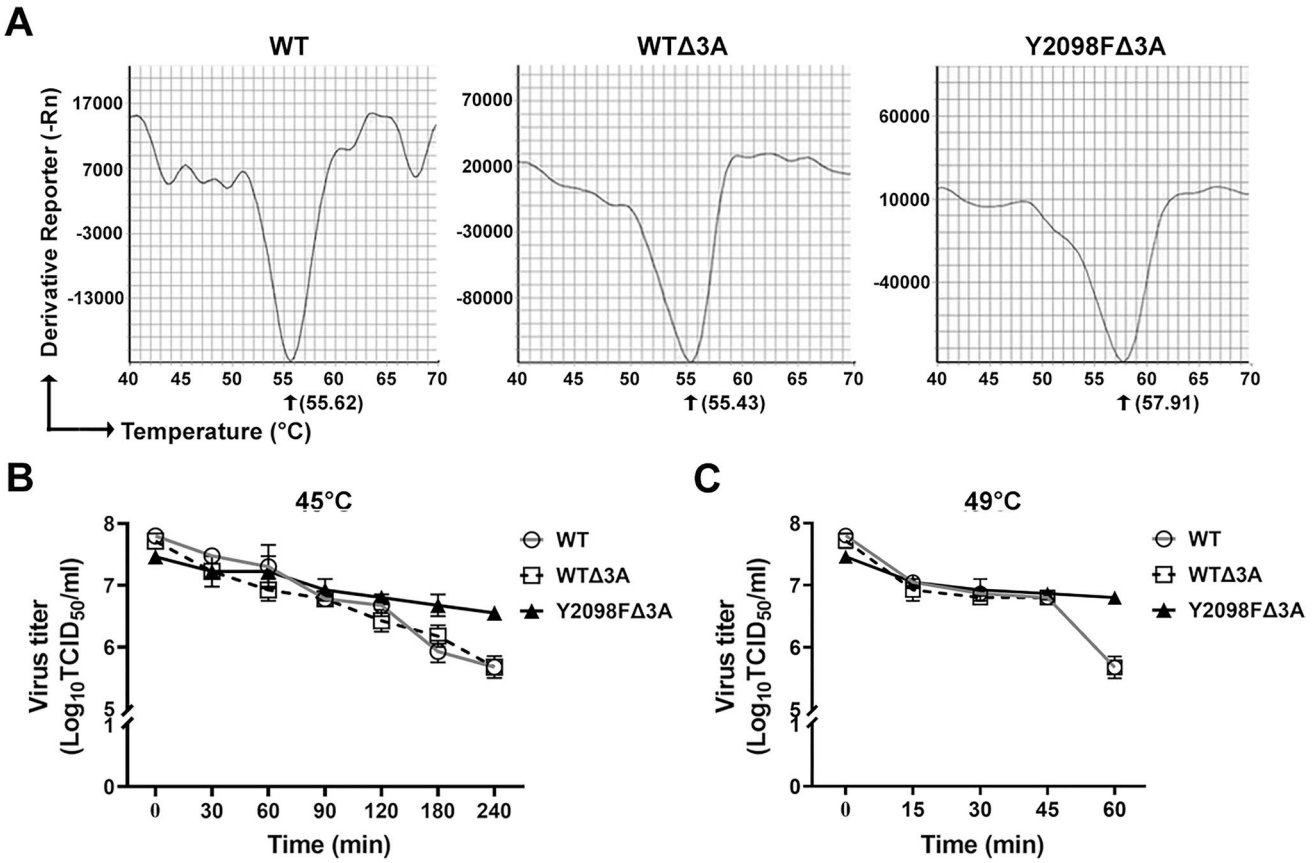

Fig. 5 Analysis of the recombinant viruses by thermofluor PaSTRy assay and thermo-inactivation assay. **A** The dissociation temperature of the wild-type (WT), WTΔ3A, and Y2098FΔ3A viruses determined by thermofluor PaSTRy, showing improved stability of Y2098FΔ3A compared to WT and WTΔ3A. **B** Thermal inactivation kinetics assays of the wild-type (WT), WTΔ3A, and Y2098FΔ3A recombinant viruses following heat treatment at 45 °C for up to 4 h. **C** Thermal inactivation kinetics assays of the wild-type (WT), WTΔ3A, and Y2098FΔ3A recombinant viruses following heat treatment at 49 °C for up to 1 h. The average virus titers at different time points were determined by $TCID_{50}$ analysis and expressed as $Log_{10}TCID_{50}$/ml

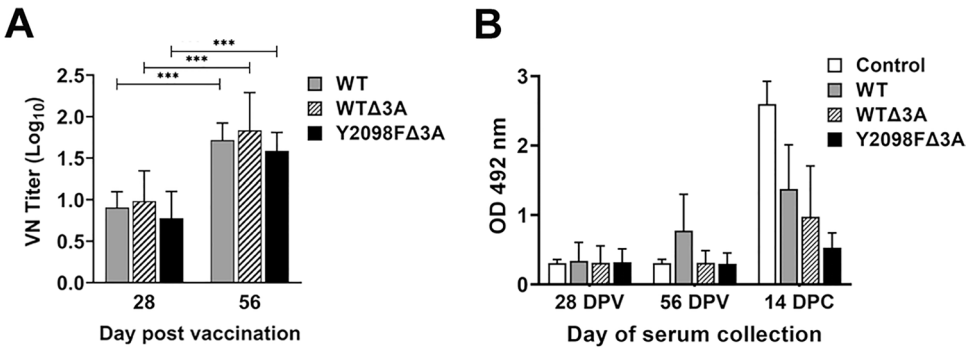

Fig. 6 Neutralizing antibody response and potency assessment by vaccination in guinea pigs. **A** The mean neutralizing titer estimated by virus neutralization test in groups of guinea pigs ($n=12$ each) immunized and boosted with inactivated wild-type (WT), WTΔ3A, and Y2098FΔ3A virus antigens with Montanide ISA 201 VG adjuvant. **B** NSP antibody response in serum collected on the 28th DPV, 56th DPV, and 14 days post-challenge on the 56th DPV, estimated by t3A indirect ELISA in the guinea pigs ($n=12$ each) immunized and boosted with inactivated wild-type, WTΔ3A, and Y2098FΔ3A virus antigens compared to the PBS-inoculated control group

protected guinea pigs, the Y2098FΔ3A immunized group showed 91% protection (Table 2).

In t3A antigen-based indirect ELISA, the 56th DPV serum samples from only the WT-vaccinated guinea pigs showed antibodies to deleted 3A region. However, on the 14th DPC, all the guinea pigs challenged with wild-type virus showed antibodies to deleted 3A region. As expected, the control group following virus challenge produced higher level of antibodies to t3A compared to vaccinated groups. In agreement with the observed higher protection, Y2098FΔ3A group showed lesser NSP antibodies (Fig. 6B).

Evaluation of competitive ELISA targeting the deleted region of 3A protein

As a companion to negative marker vaccine, cELISA to detect antibodies against FMDV NSP was developed using the recombinant t3A protein as the coating antigen and corresponding hyperimmune serum raised in rabbit as detection antibody. Using a panel of 211 bovine sera, area under the ROC curve (AUC) in the cELISA was found to be 0.964, and the standard error was 0.0175 with significance level ($p<0.0001$). The sensitivity and specificity were found to be 92.3% and 97%, respectively, at 36% cut-off PI value (Fig. 7A–C). The results were comparable to commercial PrioCHECK FMDV NS ELISA that showed sensitivity and specificity of 96.15% and 90.23%, respectively, at 50% cut-off (Fig. 7D; Table 3).

Discussion

Availability of negative marker FMD vaccine with enhanced thermostability is beneficial in FMD control program. In this study we generated and characterized a thermostable-negative marker vaccine virus candidate of FMDV serotype O (IND/R2/75) by reverse genetics approach. Additionally, a companion serological test was developed to aid in differentiation of infected animals from vaccinated animals. It was previously shown that FMDV serotype O IND-R2/1975 with 93–143 amino acids deletion in 3A and 10–37 amino acids deletion in 3B was rescuable (Biswal et al. 2015). Also, in serotype A IND40/2000, we had rescued virus with amino acid residues 87–144 deletions in 3A region (Dhanesh et al. 2020). Also, antibodies against the 3A and 3B are relatively more sensitive indicators of FMDV replication (Grubman 2005; Henderson 2005). It is reported that capsid thermostability could be improved by mutations of amino acids in the region of VP2 and VP3 proteins (Mateo et al. 2003, 2008). Based on our earlier study of immune response in cattle (Biswal et al. 2022), the specific amino acid change in

Table 2 Neutralizing antibody response and percent protection of inactivated FMDV vaccine of Y2098FΔ3A, WTΔ3A, and WT viruses in guinea pigs

SI no	Experimental group	No. of animals	Log_{10}VN titer (mean ± SD)		Number protected/number challenged	Percent protection
			28th DPV	56th DPV		
1	Y2098FΔ3A	12	0.78 ± 0.32	1.59 ± 0.22	11/12	91%
2	WTΔ3A	12	0.98 ± 0.36	1.84 ± 0.45	9/12	75%
3	WT	12	0.91 ± 0.19	1.72 ± 0.20	9/12	75%
4	PBS Control	12	0.15 ± 0.0	0.15 ± 0.0	0/12	0%

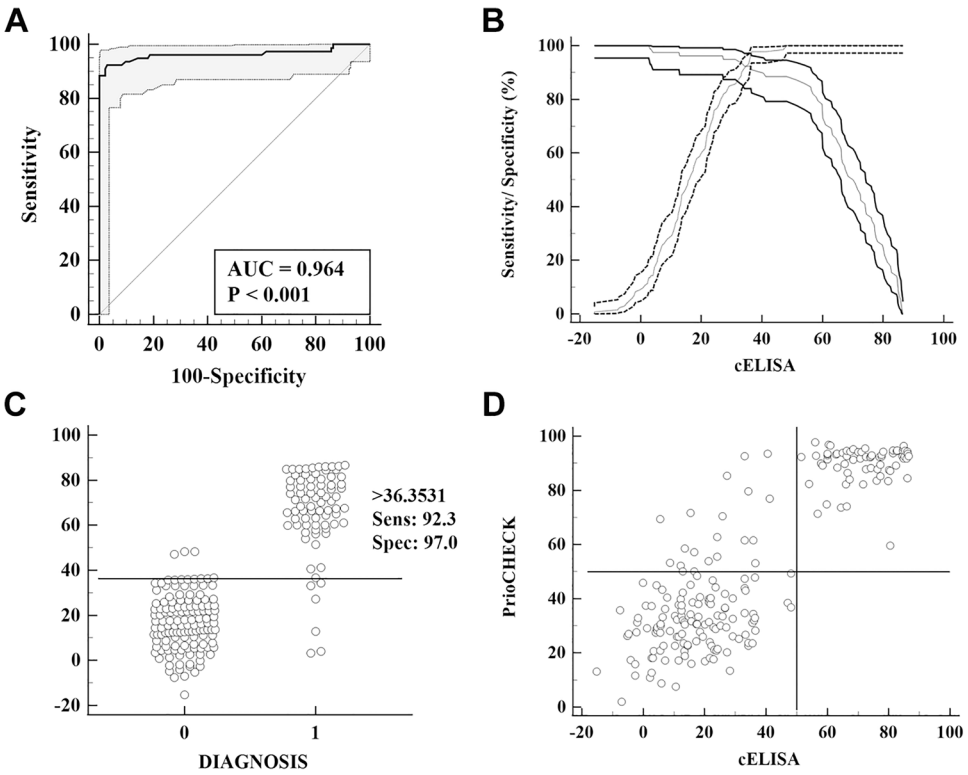

Fig. 7 Evaluation of antibodies against FMDV NSP 3A (87–144) by cELISA. **A** ROC analysis for the estimation of cut-off values of cELISA with results obtained from a total of 211 cattle sera. The gray area in the graph displays 95% confidence bounds for the ROC curve. **B** Plot of sensitivity and specificity versus criterion values with sensitivity and specificity values are displayed as percentages. **C** Interactive dot diagram visualizing the diagnostic test accuracy (0 = negative; 1 = positive). The data of the negative and positive groups are displayed as dots with the horizontal line indicating the cut-off point to distinguish between the groups. **D** Scatter diagram showing the graphical representation of the relation between commercial PrioCHECK FMDV NS ELISA and in-house developed cELISA with the reference lines at 50%

Table 3 Performance of cELISA in comparison to PrioCHECK FMDV NS test

Sample	Sample size (*n*)	In-house cELISA		PrioCHECK FMDV NS	
		Number positives (%)	Number negatives (%)	Number positives (%)	Number negatives (%)
Control	46	0 (0)	46 (100)	0 (0)	46 (100)
Vaccinated	87	3 (3.45)	84 (96.55)	13 (14.94)	74 (85.06)
Infected	78	72 (92.3)	6 (7.69)	76 (97.44)	2 (2.56)

VP2 region (Y2098F) was selected for the introduction of thermotolerant feature to the negative marker virus. Based on these reasonings, we constructed FMDV O/R2/1975 infectious cDNA clone with deletion of 58 amino acids in the C terminal of 3A, together with the thermostable mutation (Y2098F) in the VP2 region. From these, 3A portion deletion mutant viruses were rescued. The rescued negative marker viruses wild-type 3A portion deletion mutant (WTΔ3A) and thermostable mutant with 3A portion deletion (Y2098FΔ3A) were evaluated for growth characteristics, in vitro genetic stability in the cell culture, temperature stability, and immunogenicity in guinea pigs.

The growth kinetics study of recombinant viruses revealed the attainment of maximum titer by 12–18 hpi by all viruses. However, the growth of Y2098FΔ3A virus was delayed initially with lesser titer, but all the recombinant viruses attained similar titer by 24 hpi. The initial delay in the growth of Y2098FΔ3A may be due to the presence of combined effect of mutation and deletion in 3A. The antigenic profiling by western blotting showed expected band pattern for the viral structural proteins (VP3/VP1) and non-structural proteins 3A, 3B, and 3D for the recombinant viruses as well as parental wild-type virus. Previously, it was reported that VP2_S93Y mutant in serotype SAT2 virus was genetically unstable in BHK-21 cells and acquired mutation to S93H (Scott et al. 2017); we therefore analyzed the genetic stability of the rescued mutant viruses by in vitro 12 passages in BHK-21 cells. The mutants were genetically stable, retaining the introduced mutation. The thermostability of the recombinant mutant viruses was studied by thermo-inactivation assay (Scott et al. 2017), and capsid thermal stability was assessed by ThermoPaSTRy (Kotecha et al. 2016; Walter et al. 2012). Y2098FΔ3A virus showed an increase in the thermostability compared to wild type and WTΔ3A by both assays.

Guinea pigs have been used for FMDV vaccine efficacy trials (Cartwright et al. 1982). Therefore, we used the guinea pig model to evaluate the immune response of WTΔ3A and Y2098FΔ3A vaccine in comparison with wild-type virus vaccine. The deletion marker vaccines produced serum neutralizing antibody titer on the 28th and 56th DPV, comparable with wild-type virus vaccine. Further, following challenge with wild-type virus, while all the animals in control unvaccinated group developed FMD-specific lesions, wild-type and WTΔ3A antigen immunized animals showed 75% protection (9 out of 12 animals), whereas Y2098FΔ3A antigen immunized group showed 91% (11 out of 12 animals) protection. These results demonstrated an improved protection offered by the thermostable-negative marker virus antigen compared to the wild-type virus or wild-type negative marker virus antigens.

We have also developed a competitive ELISA as a companion differential diagnostic based on the deleted region of 3A protein. Screening of a representative panel of 211 bovine sera samples consists of unvaccinated FMD seronegative, FMD-vaccinated, and FMDV-infected animals using this cELISA. The cut-off for the C-ELISA was selected as > 36% PI value, and the corresponding sensitivity and specificity was found to be 92.3% and 97%, respectively, at 95% confidence interval. The results of in-house developed cELISA correlated with the results of the commercial kit. The preliminary results indicated the applicable prospects of the cELISA for DIVA.

The thermostable-negative marker FMDV serotype O vaccine strain and the companion cELISA could potentially be useful in regions where serotype O is predominantly prevalent or could be used in combination with similarly designed negative marker vaccine strains of other serotypes prevailing in the region. This study also opens the prospect of retaining the NSPs in the vaccine, yet being able to differentiate infected and vaccinated animals, since the presence of NSPs in the vaccine could potentially induce cell-mediated immune responses. Viral 3Dpol was shown to be a major cross-reactive immune determinant of FMDV, eliciting heterotypic T-cell responses in cattle (Collen et al. 1998). Also, a number of epitopes in 3A, 3B, and 3C are reported to stimulate porcine T cells (Blanco et al. 2001). Therefore, it is reasonable that retaining NSPs in the inactivated FMD vaccine will endorse cellular immune response, inducing longer duration of immunity.

In conclusion, we report a novel negative marker vaccine virus having higher thermal capsid stability, which produced protective immune response in the guinea pig model. Additionally, the competitive ELISA targeting the deleted portion of 3A protein is suitable as a companion diagnostic test for differentiation of FMD-infected and vaccinated animals. Further validation of vaccine efficacy of this thermostable-negative marker virus is required in the target natural host species, besides the required studies on its adaptability in suspension cell culture and efficiency in multivalent vaccine formulation.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00253-023-12359-w.

Acknowledgements We thank the Director, ICAR-Indian Veterinary Research Institute Izatnagar, and the Joint Director, ICAR-Indian Veterinary Research Institute Bengaluru campus, for facilitating this work. We thank the supporting staff of institute animal facility for assistance in the care and handling of animals. The first author acknowledges the fellowship and contingency grant received from ICMR JRF for her PhD program. The authors also acknowledge the SEPPIC-Air Liquide Healthcare Specialty Ingredients manufacturer, for providing Montanide ISA-201 VG adjuvant.

Author contribution SHB conceived and designed the research. HBR, VVD, MH, and UJ conducted experiments. HBR and MH collected the reagents. HBR, MH, BPS, AS, PS, and SHB analyzed data. HBR wrote the manuscript, and SHB, MH, BPS, JKB, AS, and VB edited the manuscript. All authors read and approved the manuscript.

Funding This research work was supported by grants received from ICAR-CAAST-ACLH (NAHEP/CAAST/2018–19) of the World Bank-funded National Agricultural Higher Education Project (NAHEP).

Data availability The associated data is available on request.

Declarations

Ethics statement All animal experiment protocols were carried out in compliance with the guidelines of the Institute Animal Ethics Committee (ICAR- Indian Veterinary Research Institute, Bengaluru).

Conflict of interests The authors declare that they have no competing interests.

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