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ORIGINAL ARTICLE

The carboxy-terminal half of nonstructural protein 3A is not essential for foot-and-mouth disease virus replication in cultured cell lines

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Abstract In foot-and-mouth disease (FMD)-endemic parts of the globe, control is mainly implemented by preventive vaccination with an inactivated purified vaccine. ELISAs detecting antibodies to the viral nonstructural proteins (NSP) distinguish FMD virus (FMDV)-infected animals in the vaccinated population (DIVA). However, residual NSPs present in the vaccines are suspected to be a cause of occasional false positive results, and therefore, an epitope-deleted negative marker vaccine strategy is considered a more logical option. In this study, employing a serotype Asia 1 FMDV infectious cDNA clone, it is demonstrated that while large deletions differing in size and location in the carboxy-terminal half of 3A downstream of the putative hydrophobic membrane-binding domain (deletion of residues 86-110, 101-149, 81-149 and 81-153) are tolerated by the virus without affecting its infectivity in cultured cell lines, deletions in the amino-terminal half (residues 5-54, 21-50, 21-80, 55-80 and 5-149) containing the dimerization and the transmembrane domains are deleterious to its multiplication. Most importantly, the virus could dispense with the entire carboxy-terminal half of 3A (residues 81-153) including the residues involved in the formation of the 3A-$3B_1$ cleavage junction. The rescue of a replication-competent FMDV variant carrying the largest deletion ever in 3A (residues 81-153) and the fact that the deleted region contains a series of linear B-cell epitopes inspired us to devise an indirect ELISA based on a recombinant 3A carboxy-terminal fragment and to evaluate its potential to serve as a companion diagnostic assay for differential serosurveillance if the 3A-truncated virus is used as a marker vaccine.

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Introduction

Foot-and-mouth disease (FMD) caused by FMD virus (FMDV), a member of the genus *Aphthovirus* within the family *Picornaviridae*, is one of the most difficult-to-control infectious animal viral diseases. In FMD-endemic parts of the world, effective disease control has relied mainly upon preventive vaccination with an inactivated purified vaccine containing the 146S antigens of appropriate serotypes/strains, accompanied by intensive surveillance. Detection of antibodies to viral nonstructural proteins (NSP) is currently the basis of differential serosurveillance of FMD in a vaccinated population, considering the logic that NSP antibodies are induced exclusively in case of virus replication in an infected host. However, antibodies reportedly elicited by residual NSPs present in inactivated vaccines interfere with the unequivocal identification of infected animals in the context of repeated vaccination [1–5]. More than anything else, stringent regulatory requirements to separate NSPs from the capsid antigens add to the cost of vaccine production. In addition, inclusion of the NSPs in the vaccine formulation is believed to enhance its potency and protective efficacy [6, 7]. Therefore, there is a need to develop a strategy so that infected animals are differentiated from the vaccinated uninfected ones (DIVA) more reliably. To this end, application of a modified virus carrying a deletion in a conserved immunodominant yet nonessential segment of one of its proteins as a negative marker vaccine in conjunction with a companion diagnostic assay for detecting antibodies to the deleted epitopes appears to be a rational proposition [7–9].

Viral NSP 3A, either as a determinant of virulence and host specificity or for developing a live attenuated vaccine, has been the focus of several studies [10–17]. The 3A protein of FMDV is unusually longer than that of the other picornaviruses [18]. The conserved amino-terminal (N-terminal) half contains a putative transmembrane (TM) domain (residues 59 to 76) and a dimerization domain with a hydrophobic interface (residues 25 to 44) [19, 20], thought to be important for virus multiplication. In contrast, the variable carboxy-terminal (C-terminal) half of 3A (downstream of the TM domain) is known to tolerate extensive deletions, where 10- to 20-amino-acid (aa) deletions in some of the natural outbreak strains and egg-adapted or genetically engineered derivatives of FMDV have been linked to a bovine-attenuation phenotype and marked species restriction [10–16, 21]. In a previous study, using overlapping synthetic peptides spanning the whole open reading frame of FMDV strain O1K and serum from FMDV-infected cattle, three linear B-cell epitopes were identified in the C-terminal half of 3A [22]. However, deletions engineered so far in the 3A region of the recombinant viruses have taken only the naturally observed viable limits into consideration and the precise limits of deletion tolerated by the virus is not known. Therefore, the present study was designed to construct and characterize *in vitro* several genetically modified derivatives of FMDV harbouring novel deletions within 3A, beyond those tested or observed to date, using an infectious cDNA clone of the Asia 1 IND 491/1997 strain with a full-length 3A [23]. Also, the immunoreactive domain corresponding to the longest viable deletion (81-153 aa) introduced in 3A in this study was expressed in a prokaryotic host. An indirect ELISA using the recombinant antigen (3Ac-term I-ELISA) was optimized and evaluated for its ability to serve as a DIVA-compliant diagnostic test in case such a 3A-truncated virus is adapted as a negative marker vaccine in the future.

Materials and methods

Cells, viruses and genomic cDNA clone

Three FMDV-susceptible cell lines, BHK-21 from hamster kidney and LFBK and IB-RS-2, both of pig kidney origin, were maintained in Glasgow minimum essential medium (Sigma-Aldrich, USA) supplemented with 10 % fetal bovine serum. A full-length cDNA clone of an FMDV serotype Asia 1 strain (Asia 1 IND 491/1997) assembled in the pBluescript II SK (+) vector downstream of a T7 promoter (pBlueT7Asia1IND491/1997) was used to rescue the recombinant parental virus with intact 3A and to construct all 3A-truncated derivatives [23].

Construction of 3A-truncated cDNA clones

The full-length cDNA clone pBlueT7Asia1IND491/1997 with intact 3A was used as the template for mutagenesis of the 3A region. In total, nine amplicons carrying deletions of various parts of 3A (Fig. 1) were generated by overlap

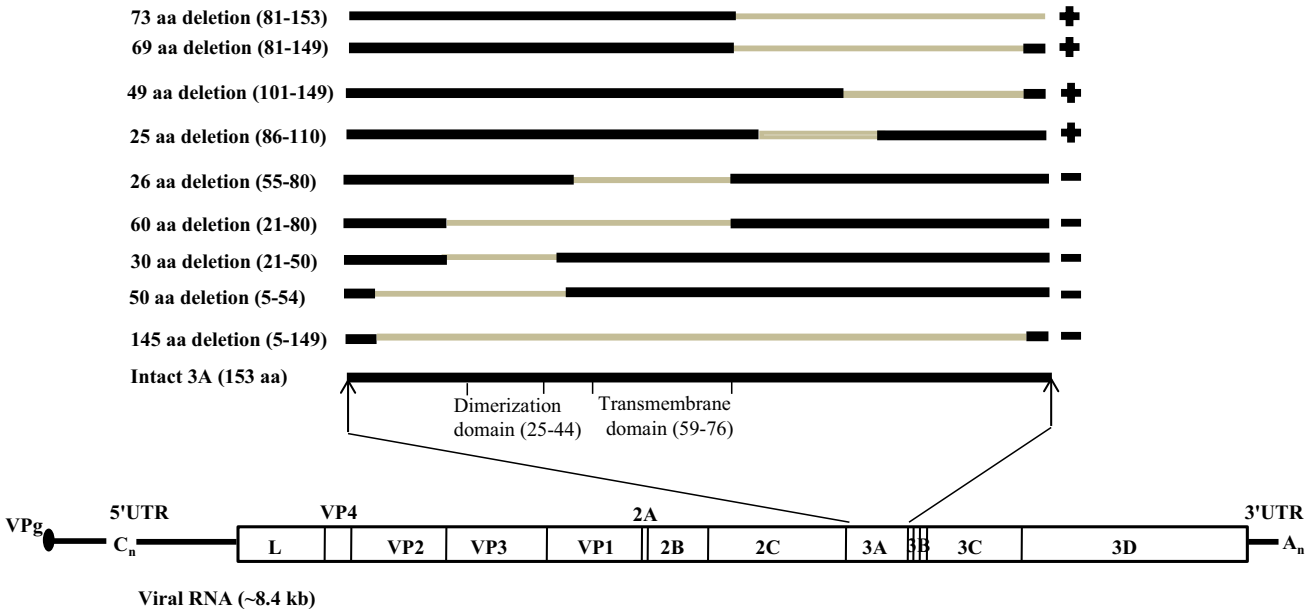

Fig. 1 Approximate location of deletion mutations engineered in the 3A region of an infectious cDNA clone of Asia 1 IND 491/1997 virus. The deletions are shown in gray shading. Successful and no rescue of virus are indicated by the symbols '+' and '−', respectively

extension PCR using a pair of common external primers, an inner pair of mutagenic primers flanking the regions of 3A to be deleted (Table 1), and KOD Hot Start DNA polymerase (Novagen, USA). The deletions were designed upstream and downstream of and encompassing the putative TM domain. The amplicons were cloned into pGEM-T Easy Vector (Promega, USA) to generate pGEMTΔ3A DF-D*Bgl*I*Not*IR plasmids, and their sequences were confirmed. Subsequently, the target regions spanning 3A were excised from the pGEMTΔ3A DF-D*Bgl*I*Not*IR plasmids and subcloned into a pBluescript II SK (+) vector carrying the genomic fragment extending from the end part of 2B to the poly(A) tail of Asia 1 IND 491/1997 virus (pBlue DF-E2) [23], using *Bgl*II and *Nar*I restriction enzymes to create pBlueΔ3A DF-E2 plasmids with specific deletions in 3A. The region encompassing 3A was excised from pBlueΔ3A DF-E2 plasmid and swapped with the corresponding fragment of pBlueT7Asia1IND491/1997 [23], using *Bgl*II and *Mlu*I restriction enzymes so that the intact 3A was replaced by different truncated forms of 3A. The 3A-truncated genomic cDNA constructs thus generated were designated as pBlueT7Asia1IND491/1997Δ3A$_{n\text{-}n}$, where 'n-n' stands for the position of residues deleted.

Rescue of recombinant viruses

Recombinant plasmids carrying the parental cDNA (pBlueT7Asia1IND491/1997) and 3A-deletions (pBlueT7Asia1IND491/1997Δ3A$_{n\text{-}n}$) were linearized and subjected to *in vitro* transcription using T7 polymerase, and the resulting RNA was used to transfect BHK-21 cell monolayers as described earlier [23]. After five sequential passages in BHK-21 cells, the working stocks were preserved for further characterization. In order to detect the FMDV structural antigens, antigen-detection ELISA was performed on supernatants derived at each passage [24]. Serial passaging was continued for an additional five rounds wherever cytopathic effect (CPE) and absorbance in antigen detection ELISA were not evident up to the fifth passage.

In vitro characterization of recombinant viruses

The *in vitro* growth kinetics of the rescued 3A-truncated viruses in relation to the parental virus were studied by constructing multi-step growth curves using virus infectivity titres ($\log_{10}$ $TCID_{50}$/ml) determined every 2 h postinfection in BHK-21 cell monolayers (Fig. 2). For this experiment, preformed BHK-21 cell monolayers were infected at a multiplicity of infection (MOI) of 0.1 and were washed thoroughly with phosphate-buffered saline (pH 6) 1 h postinfection. Plaque assays were performed as described previously [25]. The viruses rescued in BHK-21 cells were also used to infect LFBK and IB-RS-2 cell monolayers to investigate their replication ability in cell lines derived from a natural host.

In order to investigate the stability of the introduced deletions and to determine if any other mutations have accrued, RNA was extracted from the infected culture supernatant after five passages in BHK-21 cells and reverse transcribed using an oligo $d(T)_{20}$ primer. The whole genome was amplified in several overlapping fragments, and the nucleotide sequences of the rescued viruses were determined using primers described previously [26]. The nucleotide and deduced amino acid sequence alignments were performed using the CLUSTAL W algorithm available in MEGA5 software [27]. Infected cell lysates collected 6 h postinfection were resolved on 15 % SDS-PAGE, and Western blot analysis was conducted using anti-3AB guinea pig hyperimmune serum (raised earlier against the recombinant $3AB_{123}$ protein and available in the laboratory) to visualize 3A and its polyprotein precursors (Fig. 3).

Expression of 3A C-terminal fragment spanning aa residues 81 to 153 (219 bp)

The 3A C-terminal fragment (219 bp) was amplified by PCR using the full-length cDNA clone pBlueT7Asia1IND491/1997 as the template, together with suitable primers (Table 1). The 3A C-terminal amplicon was digested with restriction enzymes and then ligated to the pET-45b (+) vector (Novagen, Darmstadt, Germany). The recombinant plasmids were introduced into the *E. coli* BL21 (DE3) pLysS expression host (Novagen) by transformation. Protein expression was induced with 1 mM IPTG at 30 °C for 6 h. The yield and the size of the hexahistidine-tagged recombinant 3A C-terminal fragment were analyzed by 20 % SDS-PAGE (Fig. 4A). The immunoreactivity of the recombinant 3A C-terminal fragment was tested using anti-3AB guinea pig hyperimmune serum in Western blot (Fig. 4B). Metal affinity chromatography purification of the target protein was performed using a B-PER^{R} 6xHis fusion protein purification kit (Pierce, Rockford, USA), and the concentrated protein was stabilized with trehalose (100 mM) and protease inhibitor cocktail set III (Calbiochem, Germany) before lyophilization.

Serum samples

A panel of cattle serum samples representing naive, FMDV-infected and uninfected vaccinated animals, obtained from the serum repository of the laboratory, were used in this study.

Samples ($n = 28$) were collected from a village in Odisha state, where neither any incidence of FMD nor

Table 1 Details of primers used in this study

Primer designation	Nucleotide sequence (5′-3′)	Purpose
DF	ACCTTGGATCCGAAGAAGATCTCCGAGTCGCTC	Outer pair of common primers for overlap extension PCR
D *Bgl*I*Not*IR	GGCGCGGCCGCAGTAGCCAGCCTTAGTGGCGGC	
3A 5-149aa delF	ATTTCAATTCCTCCACAAGTTGAGGGACCCTACGCC	Inner mutagenic primers for deletion of amino acids 5-149
3A 5-149aa delR	ACTTGTGGAGGAATTGAAATCTGCTTGAAAATC	
3A 101-149 aa delF	AACATCACCCCACAAGTTGAGGGACCCTACGCC	Inner mutagenic primers for deletion of amino acids 101-149
3A 101-149 aa delR	TCAACTTGTGGGGTGATGTTTGCTTTCTCAATGTAC	
3A 86-110 aa delF	AGACAGCAGATGAAGAACCCTCTGGAGACCAGTGG	Inner mutagenic primers for deletion of amino acids 86-110
3A 86-110 aa delR	CAGAGGGTTCTTCATCTGCTGCTCCTTGCGAGTCTC	
3A 81-149 aa delF	GAGACTCGCCCACAAGTTGAGGGACCCTACGCC	Inner mutagenic primers for deletion of amino acids 81-149
3A 81-149 aa delR	TCAACTTGTGGGCGAGTCTCGCGGATCATGATC	
3A 81-153 aa delF	CCGCGAGACTCGCGGACCCTACGCCGGGCCAC	Inner mutagenic primers for deletion of amino acids 81-153
3A 81-153 aa delR	AGGGTCCGCGAGTCTCGCGGATCATGATCTC	
3A 21-80 aa delF	GGCCAACACGAAAAGAGACAGCAGATGGTGGAATGATGC	Inner mutagenic primers for deletion of amino acids 21-80
3A 21-80 aa delR	CTGCTGTCTCTTTTCGTGTTGGCCTTTCTCAATGAGG	
3A 21-50 aa delF	GGCCAACACGAAGCTTTCAACGCTCTGAAGAAAAC	Inner mutagenic primers for deletion of amino acids 21-50
3A 21-50 aa delR	ACGCTTGAAAGCTTCGTGTTGGCCTTTCTCAATGAG	
3A 55-80 aa delF	GCTTTCAAGCGTAAGAGACAGCAGATGGTGGATGATGC	Inner mutagenic primers for deletion of amino acids 55-80
3A 55-80 aa delR	CTGCTGTCTCTTACGCTTGAAAGCGCGTTGCAC	
3A 5-54 aa delF	TCAATTCCTCTGAAGGAAAACTTTGAGATTGTTGC	Inner mutagenic primers for deletion of amino acids 5-54
3A 5-54 aa delR	GTTTTCCTTCAGAGGAATTGAAATCTGCTTGAAAATC	
3A 81-153expressF	AGGATCCCAAGAGACAGCAGATGGTGG	Primers for amplification and expression of 3A aa 81-149 fragment
3A 81-153expressR	CGCGGCCGCTTACTCAACTTGTGGTTGCTCCTCCAG	

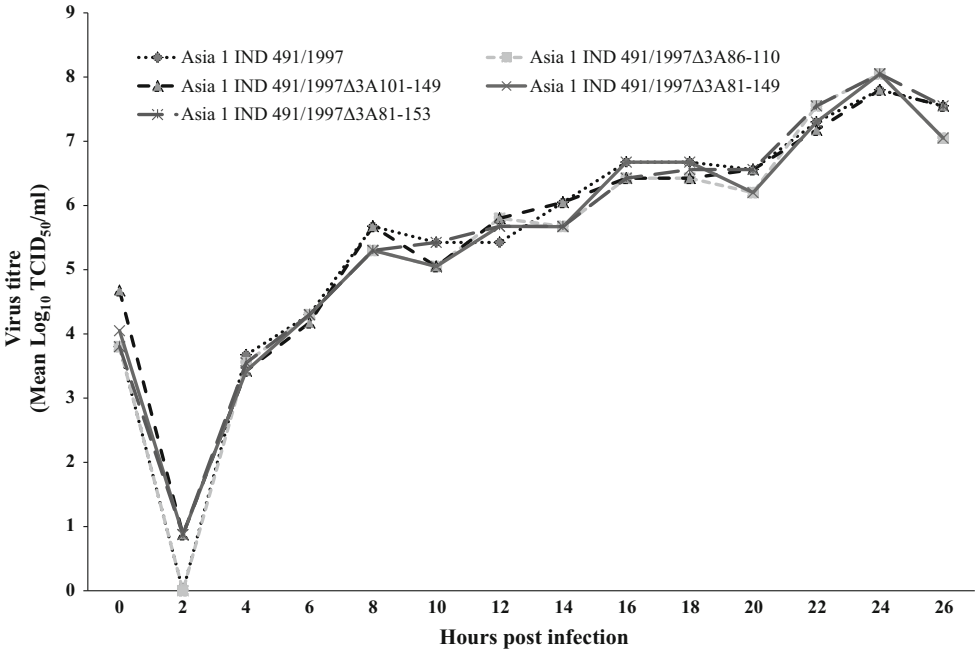

Fig. 2 Multi-step growth curves for 3A-truncated and parental viruses. The curves were constructed by plotting mean virus titres ($\log_{10}$ $TCID_{50}$/ml) as determined by endpoint dilution of infected cell culture supernatant every 2 h after low-MOI infection in BHK-21 cell monolayers

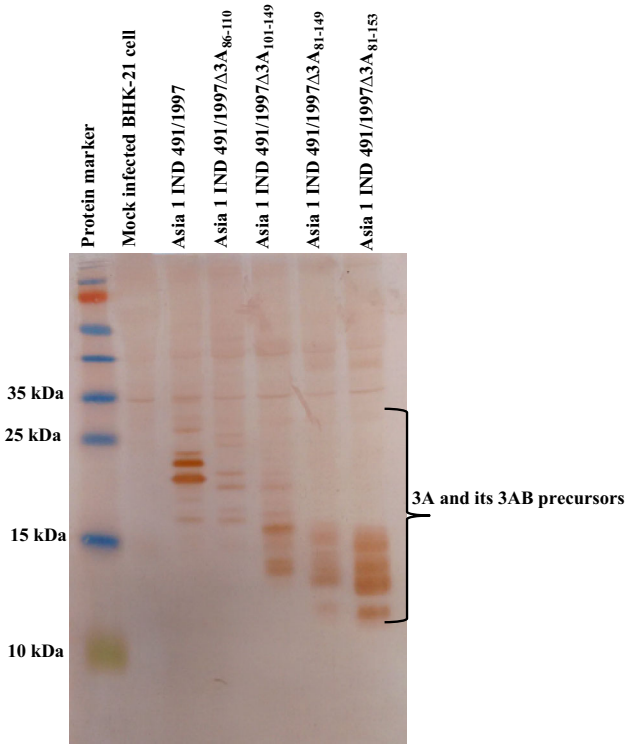

Fig. 3 Western blot analysis of BHK-21 cell lysates 6 h postinfection using anti-3AB guinea pig hyperimmune serum. The blot shows the differences in size of the 3A and its precursor polyproteins among the 3A-truncated and parental viruses

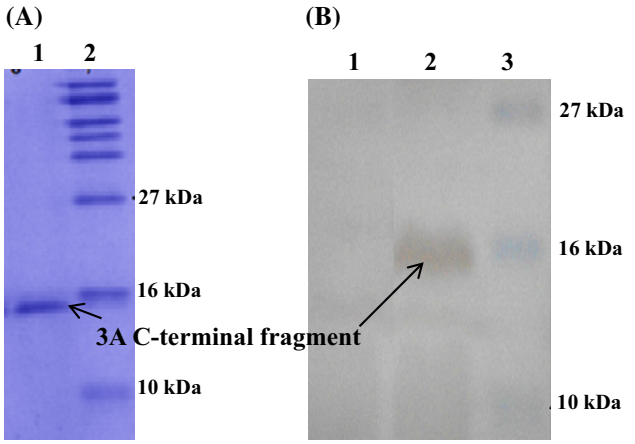

Fig. 4 (A) 20 % SDS-PAGE profile of recombinant 3A C-terminal fragment. Lane 1, affinity purified recombinant protein; lane 2, protein marker. (B) Western blot analysis of 3A C-terminal fragment expression using anti-3AB guinea pig hyperimmune serum. Lane 1, untransformed *E. coli* lysate; lane 2, IPTG-induced *E. coli* lysate; lane 3, protein marker

vaccination had been practised for over 10 years before sampling. All of those samples scored negative ($\log_{10}$ titre < 0.9) for structural protein antibodies (SP-antibodies) against the three prevalent serotypes (O, A and Asia 1) in liquid-phase blocking ELISA (LPBE), performed essentially as described earlier [5]. Additionally, commercially procured cattle serum ($n = 4$) originating from countries known to be 'FMD-free without vaccination' (Sigma-Aldrich, MO, USA and Hyclone Laboratories, UT, USA) were included. This set of 32 naive samples was used to determine the cutoff and diagnostic specificity.

Samples ($n = 81$) were obtained sequentially from two experimental calves, each vaccinated every 6 months (up to

8 boosters) with the commercial trivalent (O, A and Asia 1 strains), inactivated, oil-adjuvanted vaccines available in India. Serum samples ($n = 417$ from 72 head of cattle) collected 1 and 6 months after each round of vaccination from a regularly vaccinated, closed dairy cattle herd with no history of FMD during the 15 years prior to collection were also included in the study. All of these samples ($n = 498$) were used for determining the cutoff and diagnostic specificity.

Cattle ($n = 543$) that had shown clinical symptoms in field outbreaks (involving one of the serotypes O, A or Asia 1) were sampled. These samples, obtained 2 weeks to 4 months after the onset of symptoms, were employed for the estimation of the cutoff and diagnostic sensitivity. In addition, 174 serum samples obtained at various days postinfection (up to 1003 dpi) from four unvaccinated calves, of which two were inoculated intradermolingually with either A IND 40/2000 or Asia 1 IND 63/1972 virus, while the other two were contact infected by being cohoused with either of the inoculated animals, were used to study the post-infection kinetics of antibody evoked against the 3A C-terminal fragment.

Development of 3Ac-term I-ELISA

Pooled FMD convalescent and naïve cattle sera already in use as the internal assay controls in the r3AB3 I-ELISA [5], the validated primary screening assay in use in India, were employed as the positive and negative controls, respectively, in each run of the 3Ac-term I-ELISA. The optimum concentration of purified 3A C-terminal fragment for coating on the ELISA plate ($\sim$100 ng/well) and the dilution of serum were fixed by testing a gradient of protein concentrations against serial twofold dilutions of control sera in a checkerboard titration format. The optimized test protocol and buffer compositions for 3Ac-term I-ELISA were exactly identical to those used for r3AB3 I-ELISA [5] except that the test serum dilution was fixed at 1:10 in order to achieve the maximum separation between the optical density (OD) values of known infected and uninfected samples. The mean OD values of the positive controls (mOD_{pos}), the negative controls (mOD_{neg}), and the test samples (mOD_{sample}) were corrected by subtracting the mean OD value of the conjugate control wells (mOD_{bg}). The OD for each test sample was expressed as a percentage of the positive control using the following formula: Percent positivity (PP) value = $(mOD_{sample} - mOD_{bg})/(mOD_{pos} - mOD_{bg}) \times 100$. The samples were considered positive for NSP antibodies if the PP values were greater than 30 %. The performance of 3Ac-term I-ELISA with respect to diagnostic sensitivity and specificity was compared with that of the r3AB3 I-ELISA.

Immunization of cattle with inactivated concentrated crude parental and 3A-truncated virus antigen

In order to determine the differential antibody response induced against the C-terminal fragment of 3A in cattle inoculated with either the parental virus, which had a full-length 3A gene (Asia 1 IND 491/1997) or the negative marker virus having truncated 3A (Asia 1 IND 491/1997$\Delta 3A_{81\text{-}153}$), concentrated crude antigen was prepared without separating the NSPs. The viruses were grown in BHK-21 cell monolayer cultures in roller bottles, and the supernatants were harvested after 16 h of infection and inactivated by treatment with binary ethylenimine. The supernatants mixed with PBS were centrifuged at $3000 \times g$ for 30 min in Macrosep Advance Centrifugal Devices with 3-kDa molecular weight cutoff (Pall Life Sciences, USA) for ultrafiltration and concentration. A low molecular weight cutoff was chosen to retain the NSPs along with the capsid antigens effectively. For each virus, the collected fractions from the sample reservoirs were emulsified with Freund's complete adjuvant and infected intramuscularly into three bull calves that were seronegative in both LPBE and r3AB3 I-ELISA ($\sim$20 μg of antigen per animal). Similar antigen doses were readministered with Freund's incomplete adjuvant 21 days after the primary inoculation and 15 days after the first booster. Serum samples were collected from the preinoculated calves (0 day), 21 days after the primary inoculation, and 15 days after each of the two boosters. The collected serum samples were tested on the same day in 3Ac-term I-ELISA. This study complied with the animal ethics guidelines of the institute.

Results

Construction and rescue of 3A-truncated recombinant viruses

In this study, a series of nine 3A-truncated amplicons were generated by overlap extension PCR using the dimerization and TM domains as the points of reference. Of the nine deletions, one was designed to span nearly the full-length 3A (aa 5-149) excluding only four amino acids at each end of 3A at the polyprotein cleavage junctions, four (aa 86-110, aa 101-149, aa 81-149 and aa 81-153) were confined to the C-terminal half downstream of the TM domain, and four were designed to disrupt the TM domain and the dimerization domain, either individually or jointly in the N-terminal half of 3A protein (aa 5-54, aa 21-50, aa 21-80 and aa 55-80). A pBluescript II SK (+) vector carrying an

infectious full-length cDNA clone of FMDV Asia 1 IND 491/1997 strain under the control of a T7 promoter (pBlueT7Asia1IND491/1997) was used for introducing specific deletions in the 3A region [23] (Fig. 1). RNA transcripts derived from the 3A-truncated genomic cDNA constructs (pBlueT7Asia1IND491/1997Δ3A$_{n\text{-}n}$) were used to transfect preformed BHK-21 cell monolayers, followed by 5-10 successive passages in order to rescue and amplify the virus. Out of the nine combinations, a virus (Asia 1 IND 491/1997Δ3A$_{86\text{-}110}$, Asia 1 IND 491/1997Δ3A$_{101\text{-}149}$, or Asia 1 IND 491/1997Δ3A$_{81\text{-}149}$ and Asia 1 IND 491/1997Δ3A$_{81\text{-}153}$) could only be rescued when aa deletions were introduced in the C-terminal half of 3A downstream of the TM domain (aa 86-110, aa 101-149, aa 81-149 and aa 81-153), regardless of the size of the deletion.

Characterization of the recombinant viruses

All four viruses rescued successfully with shortened 3A displayed infectivity titres ($\log_{10}$ 7.8-8.0 $TCID_{50}$/ml) (Table 2) and growth curves in BHK-21 cell culture that were similar to those of the parental virus with intact 3A ($\log_{10}$ 7.8 $TCID_{50}$/ml) (Fig. 2). There was no relative lag observed either in the initiation of CPE or in the time taken for the production of complete cell sheet lysis for any of these rescued viruses when compared to the parental virus. Furthermore, the viruses rescued in BHK-21 cells were found to be infectious for LFBK and IB-RS-2 cell lines, with infectivity titres of $\log_{10}$ 7.5-8.2 $TCID_{50}$/ml (Table 2). The 3A-truncated recombinant viruses produced small (<1 mm diameter) and clear plaques that were indistinguishable from those of the parental virus. The engineered deletions were found to be stable in the progeny viruses after five passages in BHK-21 cells, and these viruses did not accumulate any additional mutations in the genome. The protein profile generated for the infected cell lysates in Western blot using 3AB-specific guinea pig polyclonal serum demonstrated a difference in the size of 3A and its polyprotein precursor intermediates between the 3A-truncated and the parental viruses (Fig. 3). The differential migration pattern of the bands corresponding to the 3AB region was consistent with the sizes of the deletions introduced in the 3A region. However, the individual proteins, in particular 3A and $3B_1$, need to be identified with better resolution using epitope-specific monoclonal antibodies so that that precise processing of the polyprotein into mature proteins can be elucidated.

Table 2 Infectivity titres of the rescued 3A-truncated and parental viruses in various cell lines

Virus	Titre ($\log_{10}$ $TCID_{50}$/ml)		
	BHK-21	LFBK	IB-RS-2
Asia 1 IND 491/1997	7.8	7.9	7.7
Asia 1 IND 491/1997Δ3A$_{86\text{-}110}$	8.0	8.0	8.1
Asia 1 IND 491/1997Δ3A$_{101\text{-}149}$	7.8	7.5	7.8
Asia 1 IND 491/1997Δ3A$_{81\text{-}149}$	8.0	8.2	7.9
Asia 1 IND 491/1997Δ3A$_{81\text{-}153}$	8.0	7.8	7.6

Development of companion 3Ac-term I-ELISA for the 3A-truncated marker virus

Since an FMDV mutant designed to carry a deletion of almost the entire C-terminal half of 3A could be rescued successfully and showed growth fitness comparable to that of the parental virus in cultured cell lines, the deleted fragment (residues 81-153) was expressed in *E. coli* strain BL21 (DE3) pLysS in order to develop a companion serological assay for the negative marker virus. The observed size of the His-tagged recombinant 3A C-terminal fragment in SDS-PAGE ($\sim$15.5 kDa) differed from the calculated size of $\sim$11 kDa (including the extraneous 25 aa residues derived from the vector in the form of a tag sequence) by approximately 4.5 kDa (Fig. 4). Such a discrepancy between the observed and theoretical size has also been reported previously for the recombinant 3AB and $3B_{123}$ proteins, and that has been attributed to the presence of a higher proportion of hydrophilic amino acids, causing retarded migration in the gel [5, 28, 29]. The antigenic specificity of the 3A C-terminal fragment was confirmed by Western blot using 3AB-specific guinea pig hyperimmune serum. The recombinant protein was purified to near homogeneity by nickel-IDA metal affinity chromatography for further use in 3Ac-term I-ELISA (Fig. 4). In order to establish a cutoff for qualitative judgement of infection status, a panel of serum samples from naïve ($n = 32$), vaccinated uninfected ($n = 498$) and clinically infected ($n = 543$) cattle were tested in the 3Ac-term I-ELISA. The respective absorbance values were normalized against that of the positive control serum and were expressed as percent positivity (PP) values to construct a frequency distribution chart of the PP values (Fig. 5). A 30 PP cutoff was finalized to achieve a reasonably high diagnostic sensitivity and specificity. At the 30 PP cutoff, 3Ac-term I-ELISA showed 84.8 % sensitivity, while the specificity for samples from naïve and vaccinated uninfected animals were found to be 100 % and 97.8 %, respectively. The diagnostic merit of the newly developed assay was also assessed by testing serum samples obtained from six bull calves inoculated with three doses of inactivated concentrated NSP-containing crude viral antigens derived from supernatants cultured cells infected with either parental virus Asia 1 IND 491/1997 with full-length 3A or 3A-truncated recombinant virus Asia 1 IND 491/1997Δ3A$_{81\text{-}153}$ (Table 3). Fifteen

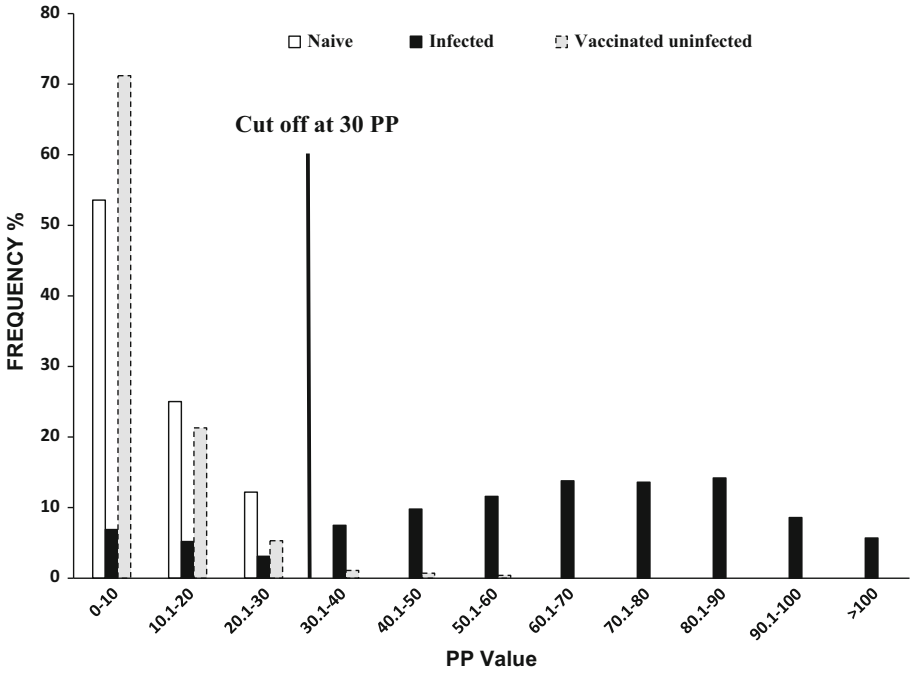

Fig. 5 Frequency distribution of percent positivity (PP) values as determined in 3Ac-term I-ELISA for serum samples derived from cattle with known FMDV infection/vaccination status. The final cutoff set for the ELISA is shown as a vertical line after the 30 PP interval

days after administration of the second booster dose, two of the three calves inoculated with the parental virus developed a detectable antibody response against the C-terminal fragment of 3A, while all three calves inoculated with the 3A-truncated negative marker virus antigen remained seronegative in 3Ac-term I-ELISA throughout the entire vaccination schedule. This difference in the induction of NSP antibodies, an important requirement for the negative marker vaccine strategy, was observed despite the fact that the virus showed comparable SP antibody titres in LPBE (Table 3).

Discussion

Both natural and laboratory-derived strains of FMDV have shown considerable flexibility in the length of NSP 3A, which tolerates large deletions or insertions, although these changes can alter host tropism, virulence and replication ability [10–13, 30]. These observations have guided further research on the role of 3A deletions in viral pathogenesis and host specificity in which precise mutations were introduced into infectious cDNA clone backbones through a reverse genetic approach [9, 14, 16, 17]. However, in previous studies, 3A deletions were restricted only to the C-terminal half, possibly considering the tolerated limits in the naturally selected FMDV strains. However, the exact limits of deletion in the C-terminal half of 3A that can be tolerated by the virus are not known.

In this exploratory study, out of the nine combinations of deletion mutations engineered in 3A, a virus (Asia 1 IND 491/1997Δ3A$_{86\text{-}110}$, Asia 1 IND 491/1997Δ3A$_{101\text{-}149}$, Asia 1 IND 491/1997Δ3A$_{81\text{-}149}$ or Asia 1 IND 491/1997Δ3A$_{81\text{-}153}$) could only be rescued when aa deletions wereintroduced in the C-terminal half of 3A downstream of the TM domain (aa 86-110, aa 101-149, aa 81-149 and aa 81-153) regardless of

Table 3 Differential NSP antibody response induced against the C-terminal fragment of 3A in cattle repeatedly immunized (3 doses) with inactivated concentrated crude viral antigens (Asia 1 IND 491/1997 with full-length 3A and 3A-truncated Asia 1 IND 491/1997Δ3A$_{81\text{-}153}$)

Virus	Cattle ID	3Ac-term I-ELISA				Log_{10} titre against serotype Asia 1 in LPBE (15 days after second booster)
		Day 0	21 days after primary dose	15 days after first booster	15 days after second booster	
Asia 1 IND 491/1997	791	–	–	–	+	2.28
	792	–	–	–	–	2.4
	793	–	–	–	+	1.8
Asia 1 IND 491/1997Δ3A$_{81\text{-}153}$	794	–	–	–	–	2.1
	795	–	–	–	–	2.4
	796	–	–	–	–	1.95

the size of the deletion. All four 3A-truncated recombinant viruses showed growth characteristics and infectivity titres comparable to that of the 3A-intact parental virus in BHK-21 cell culture. Furthermore, the viruses rescued in BHK-21 cells were found to be infectious for LFBK and IB-RS-2 cell lines (Table 2), thereby suggesting that, despite having a considerably shortened 3A, these viruses were not impaired in their ability to replicate in multiple cultured cell lines. It has been reported that synthesis of FMDV RNA and replication efficiency in primary bovine cell culture is dependent on a full-length 3A [14, 31]. Therefore, it would be interesting to study the growth efficiency of these 3A-truncated viruses in cell lines of bovine origin. An FMDV mutant carrying a deletion of residues 93-144 in 3A was the virus with the shortest length of 3A known to be rescued in any of the earlier studies [14]. Remarkably, the length of the deletion (aa 81-153) that the virus can tolerate without compromising its growth in cultured cell lines was found to be much longer (extending by 21 more aa) in this study than what has been demonstrated so far (aa 93-144). Most importantly, the virus was found to be capable of dispensing with the entire C-terminal half of 3A downstream of the TM domain, a unique region in FMDV compared to other picornaviruses. Notably, the sustainable limit of deletion extended up to the very last amino acid of the 3A C-terminus, even though it resulted in a distorted 3A-$3B_1$ polyprotein cleavage site sequence. Although it could not be established in the Western blot analysis using polyclonal serum whether 3A without an authentic junction sequence was liberated from $3B_1$ by the viral protease 3C, successful rescue of virus in the case of an unprocessed 3A-$3B_1$ can only suggest that mature processed redundant 3B copies such as $3B_2$/$3B_3$ proteins might compensate for the role played by $3B_1$ in the virus life cycle, and it is possible that 3A as a properly cleaved free protein is not essential for virus replication in cultured cells. Nevertheless, this does not undermine the probable critical role played by 3A being tethered to 3Bs in a polyprotein, either in its free or membrane-associated form, in virus replication. A direct link between a partial deletion (87-106 aa) in NSP 3A and disease attenuation in cattle has been described [16]. Since none of the natural isolates or genetically modified strains documented so far have contained 3A deletions as extensive as those rescued in this study (aa 81-153), it would be of interest to study the pathogenicity of this novel 3A-truncated virus in various susceptible host species.

In contrast, any deletion created in the N-terminal half of 3A, upstream of or involving the TM domain, was found to be detrimental to the virus, since attempts to rescue virus in three independent transfection experiments, each involving ten serial passages, failed. Any mutation other than the deletions introduced in 3A was ruled out by sequencing of the complete cDNA constructs. Neither CPE nor serotype-Asia-1-specific absorbance in antigen detection ELISA was observed with those combinations in any of the passages, suggesting that, after transfection, the RNA transcripts failed to replicate and produce infectious virions. This is not surprising given the fact that the dimerization and central membrane-binding hydrophobic domains are located in the N-terminal half of 3A, and not a single field strain with a deletion in this region has ever been reported. In an earlier study, preservation of the integrity of the predicted hydrophobic interface spanning residues 25 to 44, which is believed to be the main determinant for 3A dimerization, was demonstrated to be essential for FMDV replication [19]. A hydrophobic domain in 3A has been shown to mediate its targeting to intracellular membranes that proliferate in picornavirus-infected cells and could be responsible for the location of the viral RNA replication complex within a membrane context [32]. Taken together, the data suggest that FMDV can dispense with the entire C-terminal half (from the C-terminus of the TM domain) of 3A, at least in the cultured cell lines tested here, and show that the N-terminal half is absolutely necessary for virus replication.

The inactivated vaccines used for FMD control campaigns are not always free from the contaminating NSPs, and therefore, antibodies induced by the residual NSPs in repeatedly vaccinated uninfected animals may interfere at times with the specificity of NSP ELISAs [1–5]. An epitope-deleted negative marker vaccine along with its companion assay can enable accurate serological differentiation between infected and vaccinated animals. Although viruses with deletions in 3A have been studied for their virulence and host tropism in greater detail, their usefulness as marker vaccines has not received significant attention. Multiple linear B-cell epitopes, a major consideration in developing a diagnostic ELISA, have been mapped to the C-terminal fragment of the 3A protein [22], and one of them (residues 99-105) has been shown to be well conserved and immunodominant across FMDV serotypes [9]. The C-terminal fragment of 3A (residues 81-153) corresponding to the maximum size of the deletion in the replication-competent 3A-truncated virus rescued in this study was expressed in *E. coli* strain BL21 (DE3) pLysS in order to develop a companion ELISA (3Ac-term I-ELISA). At the outset, it may be argued that infection with unique strains carrying deletions in the 3A C-terminal region [13] might go undetected in an ELISA detecting antibodies to the 3A C-terminal fragment. However, since none of the so-far documented strains have deletions extensive enough to cover the chain of epitopes identified in the C-terminal half of 3A, this ELISA targeting multiple epitopes can be expected to have the advantage of detecting infection even with strains with partial deletions in 3A. The performance of the newly developed ELISA was evaluated against that

of the r3AB3 I-ELISA, the primary test in use for countrywide serosurveillance in India.

The diagnostic sensitivity of the r3AB3 I-ELISA (97.9 %) was found to be much higher than that of the 3Ac-term I-ELISA (84.8 %), whereas the specificity of r3AB3 I-ELISA was found to be comparable for samples from naïve animals (100 % in both tests) and lower samples from vaccinated uninfected animals (93.7 % compared to 97.8 % in 3Ac-term I-ELISA). Notably, all samples from vaccinated uninfected animals that tested positive in the 3Ac-term I-ELISA were also positive in the r3AB3 I-ELISA, thereby ruling out any possibility of anomalous presentation of the epitopes on the recombinant 3A C-terminal fragment antigen being the cause of nonspecific reaction. Theoretically, the specificity of the 3Ac-term I-ELISA for samples from vaccinated animals is expected to improve once the negative marker vaccine is put into use considering the absence of the target antigen region in the 3A-truncated virus. This assumption was supported when inactivated concentrated NSP-containing crude viral antigens derived from cell culture infected with either the parental virus with full-length 3A or 3A-truncated recombinant virus Asia 1 IND 491/1997$\Delta 3A_{81\text{-}153}$ was used to inoculate bull calves in order to assess the impact of vaccine-derived NSP impurities on the test results obtained in the 3Ac-term I-ELISA. Two of the three calves inoculated with the parental virus developed a detectable antibody response against the C-terminal fragment of 3A after administration of the second booster dose, while all three calves inoculated with the 3A-truncated negative marker virus antigen remained seronegative (Table 3). The data, although limited in scope and sample size, support application of the negative marker vaccine for reliable serosurveillance regardless of whether the vaccine contains traces of NSPs.

The lower diagnostic sensitivity of the ELISA based on the C-terminal fragment of 3A compared to 3AB polyprotein ELISA could be related either to the presence of additional highly immunogenic epitopes on the 3B protein or to a weaker, short-lived response or complete absence of seroconversion against 3A C-terminal epitopes in some of the infected animals. This presumption was substantiated by the observations made in the postinfection NSP-antibody kinetics study performed on serum samples sequentially collected from the four experimentally infected calves. While all four calves were found to be seropositive in r3AB3 I-ELISA, only three calves tested positive in 3Ac-term I-ELISA. One of the calves did not show seroconversion against the C-terminal half of 3A at any time during the period of sampling (data not shown). This is consistent with the fact that not all infected animals can be guaranteed to seroconvert against a particular NSP, and it was therefore recommended in an NSP test validation workshop that use of multiple NSP tests would enhance the chance of detecting the infected animals [33]. Furthermore, the duration of persistence of detectable NSP antibodies differed significantly between the two tests. Samples remained consistently positive in r3AB3 I-ELISA up to more than 15 months postinfection, while 3Ac-term I-ELISA showed seropositivity only up to about 4 months postinfection. Recently, the marker vaccine potential of a 3A-truncated FMDV (deletion of residues 93-144) along with a monoclonal-antibody-based blocking ELISA targeting an immunodominant epitope (residues 99–105) has been evaluated in pigs [9]. However in that study, the number of convalescent pig serum samples analyzed at 28 days postinfection was far too small to reach a conclusion about the test sensitivity or on the post-infection antibody kinetics. Although the lower diagnostic sensitivity of the 3Ac-term I-ELISA is likely to be compensated to some extent by increasing the sample size in a region with high prevalence of FMD, it renders this assay unsuitable for use in an effective marker vaccine strategy.

In summary, replication-competent FMDV engineered to carry the largest deletion ever in the NSP 3A (residues 81-153) was rescued. The data suggest that while the entire C-terminal half of 3A extending from the C-terminus of the putative membrane binding domain to the very last aa residue involved in the formation of the 3A-3B1 polyprotein cleavage junction is not essential, the N-terminal half containing the dimerization domain and the membrane-binding hydrophobic region is indispensable for virus replication and infectivity in cultured cell lines. Since the indirect ELISA (3Ac-term I-ELISA) developed as a companion assay for the 3A-truncated virus to detect antibodies evoked against the C-terminal fragment of 3A showed considerably lower diagnostic sensitivity than the r3AB3 I-ELISA, the primary NSP-antibody screening assay in use in the country, it cannot be used as a reliable serosurveillance tool in the disease control programme.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Mackay DK, Forsyth MA, Davies PR, Berlinzani A, Belsham GJ, Flint M, Ryan MD (1998) Differentiating infection from vaccination in foot and mouth disease using a panel of recombinant non-structural proteins in ELISA. Vaccine 16:446–459
2. Bergmann IE, Malirat V, Neitzert E, Beck E, Panizzutti N, Sanchez C, Falczuk A (2000) Improvement of a serodiagnostic strategy for foot and mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA 3ABC with an enzyme linked immunoelectrotransfer blot assay. Arch Virol 145:473–489

3. Lee F, Jong M, Yang D (2006) Presence of antibodies to non-structural proteins of foot-and-mouth disease virus in repeatedly vaccinated cattle. Vet Microbiol 115:14–20
4. Robiolo B, Seki C, Fondevilla N, Grigera P, Scodeller E, Periolo O, La Torre J, Mattion N (2006) Analysis of the immune response to FMDV structural and non-structural proteins in cattle in Argentina by the combined use of liquid phase and 3ABC-ELISA tests. Vaccine 24:997–1008
5. Mohapatra JK, Pandey LK, Sanyal A, Pattnaik B (2011) Recombinant non-structural polyprotein 3AB-based serodiagnostic strategy for FMD surveillance in bovines irrespective of vaccination. J Virol Methods 177:184–192
6. Pena L, Moraes MP, Koster M, Burrage T, Pacheco JM, Segundo FD, Grubman MJ (2008) Delivery of a foot-and-mouth disease virus empty capsid subunit antigen with nonstructural protein 2B improves protection of swine. Vaccine 26:5689–5699
7. Uddowla S, Hollister J, Pacheco JM, Rodriguez LL, Rieder E (2012) A safe foot-and-mouth disease vaccine platform with two negative markers for differentiating infected from vaccinated animals. J Virol 86:11675–11685
8. Fowler VL, Bashiruddin JB, Maree FF, Mutowembwa P, Bankowski B, Gibson D, Cox S, Knowles N, Barnett PV (2011) Foot-and-mouth disease marker vaccine: cattle protection with a partial VP1 G-H loop deleted virus antigen. Vaccine 29:8405–8411
9. Li P, Lu Z, Bai X, Li D, Sun P, Bao H, Fu Y, Cao Y, Chen Y, Xie B, Yin H, Liu Z (2014) Evaluation of a 3A-truncated foot-and-mouth disease virus in pigs for its potential as a marker vaccine. Vet Res 45:51
10. Giraudo AT, Sagedahl A, Bergmann IE, La Torre JL, Scodeller EA (1987) Isolation and characterization of recombinants between attenuated and virulent aphthovirus strains. J Virol 61:419–425
11. Giraudo AT, Beck E, Strebel K, de Mello PA, La Torre JL, Scodeller EA, Bergmann IE (1990) Identification of a nucleotide deletion in parts of polypeptide 3A in two independent attenuated aphthovirus strains. Virology 177:780–783
12. Beard CW, Mason PW (2000) Genetic determinants of altered virulence of Taiwanese foot-and-mouth disease virus. J Virol 74:987–991
13. Knowles NJ, Davies PR, Henry T, O'Donnell V, Pacheco JM, Mason PW (2001) Emergence in Asia of foot-and-mouth disease viruses with altered host range: characterization of alterations in the 3A protein. J Virol 75:1551–1556
14. Pacheco JM, Henry TM, O'Donnell VK, Gregory JB, Mason PW (2003) Role of nonstructural proteins 3A and 3B in host range and pathogenicity of foot-and-mouth disease virus. J Virol 77:13017–13027
15. Li S, Gao M, Zhang R, Song G, Song J, Liu D, Cao Y, Li T, Ma B, Liu X, Wang J (2010) A mutant of infectious Asia 1 serotype foot-and-mouth disease virus with the deletion of 10-amino-acid in the 3A protein. Virus Genes 41:406–413
16. Pacheco JM, Gladue DP, Holinka LG, Arzt J, Bishop E, Smoliga G, Pauszek SJ, Bracht AJ, O'Donnell V, Fernandez-Sainz I, Fletcher P, Piccone ME, Rodriguez LL, Borca MV (2013) A partial deletion in non-structural protein 3A can attenuate foot-and-mouth disease virus in cattle. Virology 446:260–267
17. Gladue DP, O'Donnell V, Baker-Bransetter R, Pacheco JM, Holinka LG, Arzt J, Pauszek S, Fernandez-Sainz I, Fletcher P, Brocchi E, Lu Z, Rodriguez LL, Borca MV (2014) Interaction of foot-and-mouth disease virus nonstructural protein 3A with host protein DCTN3 is important for viral virulence in cattle. J Virol 88:2737–2747
18. Kitamura N, Semler BL, Rothberg PG, Larsen GR, Adler CJ, Dorner AJ, Emini EA, Hanecak R, Lee JJ, van der Werf S, Anderson CW, Wimmer E (1981) Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature 291:547–553
19. Gonzalez-Magaldi M, Postigo R, de la Torre BG, Vieira YA, Rodriguez-Pulido M, Lopez-Vinas E, Gomez-Puertas P, Andreu D, Kremer L, Rosas MF, Sobrino F (2012) Mutations that hamper dimerization of foot-and-mouth disease virus 3A protein are detrimental for infectivity. J Virol 86:11013–11023
20. Gonzalez-Magaldi M, Martin-Acebes MA, Kremer L, Sobrino F (2014) Membrane topology and cellular dynamics of foot-and-mouth disease virus 3A protein. PLoS One 9:e106685
21. Sagedahl A, Giraudo AT, De Mello PA, Bergmann IE, La Torre JL, Scodeller EA (1987) Biochemical characterization of an aphthovirus type C3 strain Resende attenuated for cattle by serial passages in chicken embryos. Virology 157:366–374
22. Hohlich BJ, Wiesmuller KH, Schlapp T, Haas B, Pfaff E, Saalmuller A (2003) Identification of foot-and-mouth disease virus-specific linear B-cell epitopes to differentiate between infected and vaccinated cattle. J Virol 77:8633–8639
23. Mohapatra JK, Pandey LK, Pattnaik B (2014) RNA structure disrupting G_{320}-T transversion within the short fragment of the 5′ untranslated region prevents rescue of infectious foot-and-mouth disease virus. J Virol Methods 196:100–103
24. Bhattacharya S, Pattnaik B, Venkataramanan R (1996) Development and application of sandwich enzyme-linked immunosorbent assay (ELISA) for type identification of foot and mouth disease (FMD) virus in direct field materials. Indian J Anim Sci 66:1–9
25. Bachrach HL, Callis JJ, Hess WR, Patty RE (1957) A plaque assay for foot-and-mouth disease virus and kinetics of virus reproduction. Virology 4:224–236
26. Mohapatra JK, Sanyal A, Hemadri D, Tosh C, Biswas S, Knowles NJ, Rasool TJ, Bandyopadhyay SK, Pattnaik B (2008) Comparative genomics of serotype Asia 1 foot-and-mouth disease virus isolates from India sampled over the last two decades. Virus Res 136:16–29
27. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739
28. He C, Wang H, Wei H, Yan Y, Zhao T, Hu X, Luo P, Wang L, Yu Y (2010) A recombinant truncated FMDV 3AB protein used to better distinguish between infected and vaccinated cattle. Vaccine 28:3435–3439
29. Mohapatra AK, Mohapatra JK, Pandey LK, Sanyal A, Pattnaik B (2014) Diagnostic potential of recombinant nonstructural protein 3B to detect antibodies induced by foot-and-mouth disease virus infection in bovines. Arch Virol 159:2359–2369
30. Li P, Bai X, Cao Y, Han C, Lu Z, Sun P, Yin H, Liu Z (2012) Expression and stability of foreign epitopes introduced into 3A nonstructural protein of foot-and-mouth disease virus. PLoS One 7:e41486
31. O'Donnell VK, Pacheco JM, Henry TM, Mason PW (2001) Subcellular distribution of the foot-and-mouth disease virus 3A protein in cells infected with viruses encoding wild-type and bovine-attenuated forms of 3A. Virology 287:151–162
32. Doedens JR, Giddings TH Jr, Kirkegaard K (1997) Inhibition of endoplasmic reticulum-to-Golgi traffic by poliovirus protein 3A: genetic and ultrastructural analysis. J Virol 71:9054–9064
33. Brocchi E, Bergmann IE, Dekker A, Paton DJ, Sammin DJ, Greiner M, Grazioli S, De Simone F, Yadin H, Haas B, Bulut N, Malirat V, Neitzert E, Goris N, Parida S, Sørensen K, De Clercq K (2006) Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus. Vaccine 24:6966–6979