Virus Research 243 (2018) 36–43

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Mutational analysis of foot and mouth disease virus nonstructural polyprotein 3AB-coding region to design a negative marker virus

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ARTICLE INFO

Keywords:
FMD virus
3AB deletion
Negative marker virus
Indirect ELISA
DIVA

ABSTRACT

Inactivated purified whole virus vaccines are used for control of foot and mouth disease (FMD). ELISAs detecting antibodies to the nonstructural proteins (NSP), a marker of infection, are primarily used to differentiate FMD virus (FMDV) infected from vaccinated animals (DIVA). However, such DIVA assays have a limitation to their specificity since residual NSPs present in the relatively impure vaccines are suspected to induce an NSP-antibody response in the repeatedly vaccinated animals. Epitope-deleted negative marker vaccine strategy seems to have an advantage over the conventional vaccines in identifying the infected animals with accuracy. NSP 3AB contains an abundance of immunodominant B-cell epitopes of diagnostic importance. This study addresses the feasibility of producing 3AB-truncated FMDV mutant as a potential negative marker vaccine candidate. An infectious cDNA clone of FMDV serotype Asia 1 strain was used to engineer an array of deletion mutations in the established antigenic domain of 3AB. The maximum length of deletion tolerated by the virus was found to be restricted to amino acid residues 87–144 in the C-terminal half of 3A protein along with deletion of the first two copies of 3B peptide. The 3AB-truncated marker virus (Asia 1 IND 491/1997Δ$3A_{87\text{-}144}3B_{1,2}$+FLAG) demonstrated infectivity titres comparable to that of the parental virus in BHK-21 (log_{10} 7.42 $TCID_{50}$/ml) and LFBK-$\alpha_V\beta_6$ (log_{10} 8.30 $TCID_{50}$/ml) cell monolayer culture. The protein fragment corresponding to the viable deletion in the 3AB region was expressed in a prokaryotic system to standardize a companion assay ($3A_{87\text{-}153}3B_{1,2}$ I-ELISA) for the negative marker virus which showed reasonably high diagnostic sensitivity (96.9%) and specificity (100% for naïve and 97.1% for uninfected vaccinated samples). The marker virus and its companion ELISA designed in this study provide a basis to devise a marker vaccine strategy for FMD control.

1. Introduction

Foot and mouth disease (FMD), caused by FMD virus (FMDV) of the genus *Aphthovirus* within the family *Picornaviridae*, is a highly transmissible viral disease of livestock having considerable economic impact. Preventive vaccination with inactivated purified whole-virus vaccine accompanied by intensive surveillance has proved effective in control of the disease. Since the inactivated vaccine antigens do not replicate and therefore are not expected to induce antibodies against the viral nonstructural proteins (NSP), assays detecting NSP-antibody to differentiate infected from vaccinated animals (DIVA) have gained wide acceptance (Mackay et al., 1998). However, presence of NSP-antibodies in animals vaccinated repeatedly with vaccines containing residual NSPs is thought to interfere with the unequivocal screening of infected animals, thus warranting search for a more reliable DIVA strategy (Lee et al., 2006; Mackay et al., 1998; Mohapatra et al., 2011; Robiolo et al., 2006). Intrinsic absence of an immunogenic region in a genetically modified negative marker vaccine virus and use of its companion assay targeting antibodies induced against the deleted epitopes could overcome the impediment put by the vaccines contaminated with the NSPs in the path of accurate serological identification of the FMDV infected animals among the vaccinated ones (Behura et al., 2016; Fowler et al., 2011; Li et al., 2014; Uddowla et al., 2012).

A consensus opinion in favour of the NSP 3ABC as the most suitable target for devising DIVA-compliant serological assays has emerged over the years (Mackay et al., 1998; Brocchi et al., 2006). In a previous study, using overlapping synthetic peptides spanning the whole open reading frame of FMDV strain O1K, a series of FMDV strain-independent infection-specific linear B-cell epitopes having diagnostic value were identified in the carboxy-terminal (C-terminal) half of 3A and $3B_{1,2}$ proteins (Hohlich et al., 2003). FMDV is unique among the picornaviruses in that it encodes an unusually longer 3A and three

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http://dx.doi.org/10.1016/j.virusres.2017.10.010
Received 14 September 2017; Received in revised form 9 October 2017; Accepted 13 October 2017
Available online 14 October 2017

tandem copies of genome-linked 3B (VPg) protein (Forss and Schaller, 1982; Kitamura et al., 1981). More interestingly, FMDV genome displays amazing flexibility in the length of 3A and copy number of 3B protein. The 18 amino acid (aa) long (residues 59–76) hydrophobic domain in the central part of 3A protein (Gonzalez-Magaldi et al., 2012) is thought to anchor the 3A protein with cellular membranes thereby mediating the relocation of the RNA replication machinery to the cellular membranes (Xiang et al., 1998). Extensive deletions in 3A downstream of this hydrophobic region in the outbreak strains, in the alternative host-adapted strains or in the genetically engineered viruses have been linked to altered virulence and host tropism of the virus (Beard and Mason, 2000; Giraudo et al., 1990; Pacheco et al., 2003). The role of VPg in priming RNA synthesis has been studied in various picornaviruses (Wimmer, 1982). Although there are no reports of any natural FMDV strains with fewer than three copies of 3B peptide apart from a Korean strain having a deletion of 23 residues in $3A3B_1$ region (Park et al., 2016), it has been established that a single VPg peptide is sufficient to support virus replication *in vitro* (Falk et al., 1992; Pacheco et al., 2003).

The largest viable deletion in 3AB region ever has been shown to be 93–144 residues in 3A along with deletion of the first two copies of 3B peptide in a genetically engineered FMDV derivative (Pacheco et al., 2003). However, the maximum acceptable limits of deletion of residues spanning 3AB region without impairing virus viability *in vitro* is not known. In a couple of studies in our laboratory, deletions introduced in the 3AB region have been exploited to develop negative marker viruses and their companion ELISAs. While one study involving 3A-truncated marker virus (deletion of residues 81–153) ended up in developing an ELISA having insufficient sensitivity (Behura et al., 2016), the other was based on partial deletion within the 3AB polyprotein (Biswal et al., 2015). Considering the presence of multiple immunodominant B-cell epitopes in the polyprotein 3AB and its amenability to genetic alterations, both essential prerequisites to select a target for negative marker vaccine strategy, we made an attempt to integrate various combinations of deletions in 3A and 3B region on an infectious cDNA clone backbone (Mohapatra et al., 2014b) in order to determine the maximum permissible extent of deletion in the 3AB region. Subsequently, an indirect ELISA ($3A_{87\text{-}153}3B_{1,2}$ I-ELISA) was developed using recombinant antigen corresponding to the deleted segment in the marker virus and its performance was evaluated in relation to the r3AB3 I-ELISA (Mohapatra et al., 2011), the validated primary assay used in the country for FMD serosurveillance.

2. Materials and methods

2.1. Cells, viruses and genomic cDNA clones

Two FMDV-susceptible cell lines, BHK-21 of hamster kidney origin and LFBK-$\alpha_v\beta_6$ of pig kidney origin, were maintained in Glasgow minimum essential medium (GMEM) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum. Two cDNA clones (pBlueT7Asia1IND491/1997 carrying FMDV complete genome (Mohapatra et al., 2014b) and pBlueT7Asia1IND491/1997**Δ**$3A_{81\text{-}149}$ carrying viable 3A-truncated genome having deletion of aa residues 81–149 in 3A (Behura et al., 2016)), derived from a serotype Asia 1 strain (Asia 1 IND 491/1997) and assembled in the pBluescript II SK (+) vector downstream of T7 promoter, were used to construct all 3AB-truncated mutants in this study.

2.2. Construction of 3AB-truncated cDNA clones

The cDNA clones pBlueT7Asia1IND491/1997 (Mohapatra et al., 2014b) and pBlueT7Asia1IND491/1997**Δ**$3A_{81\text{-}149}$ (Behura et al., 2016) served as the initial templates to generate amplicons harbouring deletion varying in size and location in the 3AB region by overlap extension PCR (OE-PCR) (Fig. 1). For OE-PCR, KOD hot start DNA polymerase

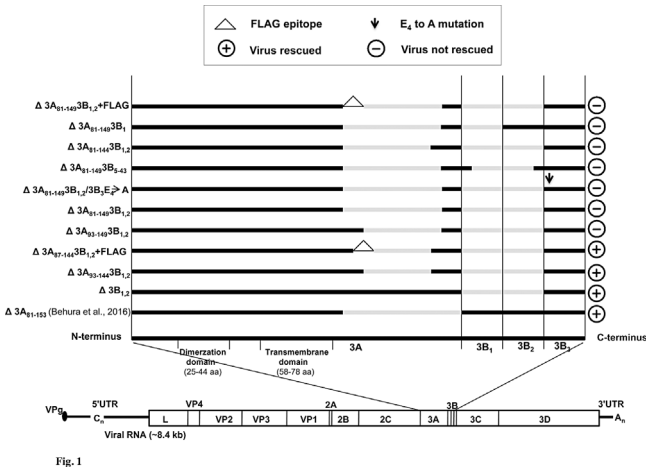

Fig. 1

Fig. 1. Approximate location and size of deletion mutations (shown in gray shade) introduced in the 3AB region of FMDV Asia 1 IND 491/1997 virus and their effect on virus rescue. The respective modifications in the cDNA constructs are indicated on the left in which 'Δ' stands for deletion and the subscripts next to the name of the proteins suggest the residues deleted. Ten combinations of deletion mutants were constructed in this study, while one ($\Delta 3A_{81\text{-}153}$) was rescued in an earlier study (Behura et al., 2016).

(Novagen, USA), a pair of common external primers (located at 2B and 3C region) and overlapping inner pair of mutagenic primers flanking the regions to be deleted were used (Table 1). The deletions were designed so as to include the first two copies of 3B protein alone or a variable length of sequence downstream of the putative hydrophobic membrane binding domain in 3A along with either the first or the first two copies of 3B protein (Fig. 1). The PCR-amplicons were cloned into pGEM-T Easy vector (Promega, USA) to generate pGEMTΔ3AB 2B*Bgl*IIF-3C*Nar*IR plasmids and their sequences were confirmed. Subsequently, the target regions spanning 3AB were excised and subcloned into pBluescript II SK (+) vector carrying the genomic fragment from end part of 2B to the poly(A) tail of Asia1 IND 491/1997 virus (pBlue DF-E2) (Mohapatra et al., 2014b) using *Bgl*II and *Nar*I restriction enzymes to create pBlueΔ3AB DF-E2 plasmids with specific deletions in 3AB region. The region encompassing 3AB was cut from pBlueΔ3AB DF-E2 plasmid and swapped with the corresponding fragment of pBlueT7Asia1IND491/1997 (Mohapatra et al., 2014b) using *Bgl*II and *Mlu*I restriction enzyme sites naturally present in FMDV genome to create 3AB-truncated genomic cDNA constructs. Those constructs were designated as pBlueT7Asia1IND491/1997$\Delta 3A_{n\text{-}n}3B_{n,n}$, where 'n-n' stands for the position of aa residues deleted and 'n,n' denotes the copies of 3B deleted.

The cDNA clone pBlueT7Asia1IND491/1997**Δ**$3A_{81\text{-}149}$ (Behura et al., 2016) was used as template to generate three constructs (pBlueT7Asia1IND491/1997$\Delta 3A_{81\text{-}149}3B_{1,2}$, pBlueT7Asia1IND491/1997$\Delta 3A_{81\text{-}149}3B_{5\text{-}43}$ and pBlueT7Asia1IND491/1997$\Delta 3A_{81\text{-}149}3B_1$). One of those cDNA clones pBlueT7Asia1IND491/1997$\Delta 3A_{81\text{-}149}3B_{1,2}$ was further used as template to introduce a point mutation (E_4 to A i.e., Glutamic Acid to Alanine) at the 4th codon (codon '$GA_{11}A$' to 'GCA') of $3B_3$ to generate pBlueT7Asia1IND491/1997$\Delta 3A_{81\text{-}149}3B_{1,2}/3B_3E_4 \rightarrow A$ using QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) so that the 3A-$3B_3$ junction conforms to the 3A-$3B_1$ junction sequence in the absence of the $3B_1$ and $3B_2$ peptides. Another cDNA construct harbouring deletion of first two copies of 3B protein (pBlueT7Asia1IND491/1997$\Delta 3B_{1,2}$) was engineered using full-length cDNA clone pBlueT7Asia1IND491/1997 as template and subsequently that construct was used as the template to introduce various combinations of deletion/insertion mutation in the 3A region by OE-PCR (pBlueT7Asia1IND491/1997$\Delta 3A_{93\text{-}149}3B_{1,2}$, pBlueT7Asia1IND491/1997$\Delta 3A_{93\text{-}144}3B_{1,2}$, pBlueT7Asia1IND491/1997$\Delta 3A_{81\text{-}144}3B_{1,2}$, pBlueT7Asia1IND491/1997$\Delta 3A_{87\text{-}144}3B_{1,2}$+FLAG, and pBlueT7Asia1IND491/1997$\Delta 3A_{81\text{-}149}3B_{1,2}$+FLAG). Finally, a set of ten 3AB-truncated cDNA