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Marker vaccine potential of foot-and-mouth disease virus with large deletion in the non-structural proteins 3A and 3B

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious, economically important disease of transboundary importance. Regular vaccination with chemically inactivated FMD vaccine is the major means of controlling the disease in endemic countries like India. However, the traditional inactivated vaccines may sometimes contain traces of FMD viral (FMDV) non-structural protein (NSP), therefore, interfering with the NSP-based serological discrimination between infected and vaccinated animals. The availability of marker vaccine for differentiating FMD infected from vaccinated animals (DIVA) would be crucial for the control and subsequent eradication of FMD in India. In this study, we constructed a negative marker FMDV serotype O virus (vaccine strain O IND R2/1975), containing dual deletions of amino acid residues 93–143 and 10–37 in the non-structural proteins 3A and 3B, respectively through reverse genetics approach. The negative marker virus exhibited similar growth kinetics and plaque morphology in cell culture as compared to the wild type virus. In addition, we also developed and evaluated an indirect ELISA (I-ELISA) targeted to the deleted 3AB NSP region (truncated 3AB) which could be used as a companion differential diagnostic assay. The diagnostic sensitivity and specificity of the truncated 3AB I-ELISA were found to be 95.5% and 96%, respectively. The results from this study suggest that the availability negative marker virus and companion diagnostic assay could open a promising new avenue for the application of DIVA compatible marker vaccine for the control of FMD in India.

1. Introduction

Foot-and-mouth disease is an extremely contagious viral disease of cloven hoofed domesticated and wild ungulates. The disease is endemic in Asia, Africa and parts of the South America where it has a high negative economic impact on animal health, productivity and international trade [1]. The etiological agent, FMD virus (FMDV) is the prototype member of the genus *Aphthovirus* in the family *Picornaviridae* [2]. The positive sense single stranded RNA genome of FMDV is protected by an icosahedral capsid containing 60 copies of each of the four structural proteins, VP4, VP2, VP3 and VP1. The viral genome encodes a single polyprotein that is post-translationally cleaved by virus-encoding proteases to yield four structural and 10 non-structural proteins (NSP) (L, 2A, 2B, 2C, 3A, $3B_{1-3}$, 3C and 3D) [3].

FMD is controlled primarily by vaccination. The current vaccine against FMD consists of chemically inactivated whole virus preparation that is formulated with either mineral oil or aluminium hydroxide adjuvant depending upon the target species [4]. Although the current vaccines can prevent the clinical signs and limit further spread of the disease, they do not induce sterile immunity [5,6]. Therefore, the possibility of FMD infection in the vaccinated population necessitates methods to identify these animals. Currently this is achieved by NSP-antibody based serological assays which permit differentiation of infected from vaccinated animals [7]. However, vaccine preparations, depending upon their sources, can contain traces of NSPs, which makes it difficult to detect infection in repeatedly vaccinated populations [8–10].

An alternative approach resides in the concept of marker vaccine against FMDV which could be engineered to exclude one or

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more viral non-structural proteins or parts thereof (negative marker vaccine). By use of the negative marker vaccine, infected animals would therefore generate serological profiles different to those of the vaccinated animals, which would permit the identification of infected animals through a companion diagnostic assay (DIVA tests). Marker vaccines and companion DIVA tests have been used successfully for the control and eradication of numerous animal diseases [11–13].

In India, a vaccination-based FMD-control programme was launched in 2003–04 with an aim of creating disease-free zones [14]. In this context, in order to have information on the level of FMDV infection in domesticated large ruminants, irrespective of vaccination, national FMD serosurveillance is being carried out by determining seroconversion against 3AB3 NSP using an in-house 3AB3 protein-based indirect ELISA [15]. Considering this background, the current study was undertaken to generate a negative marker vaccine strain against FMDV containing dual deletions of amino acid residues 93–143 and 10–37 in the non-structural protein 3A and 3B respectively, using a full-genome length infectious cDNA clone for FMDV serotype O vaccine strain O IND R2/1975. The marker virus exhibited similar growth kinetics, plaque morphology in BHK-21 cell culture as compared to the wild type virus. In addition, we also developed and evaluated an indirect ELISA targeted to the deleted 3AB NSP region (truncated 3AB) which could differentiate animals infected with the wild type viruses from those vaccinated with the new negative marker vaccines.

2. Materials and methods

2.1. Cells, viruses and plasmid

FMDV susceptible cell line BHK-21 was propagated in Glasgow minimum essential medium (GMEM, Sigma, USA) supplemented with 10% foetal bovine serum (FBS). Virus stocks were prepared and titrated in BHK-21 cells using the plaque assay [16].

FMDV serotypes O vaccine strain O IND R2/1975 was obtained from the national FMD virus repository maintained at ICAR-Project Directorate on Foot-and-mouth disease, Mukteswar, India.

FMDV serotype O IND R2/1975 infectious cDNA clone ($pO^{R2/1975}$) was used as donor plasmid for the engineering of negatively marked FMDV. The construction of full-length infection cDNA clone for FMDV O IND R2/1975 has been described earlier [17].

2.2. Construction of genome-length infectious cDNA clone containing dual deletions in 3A and 3B NSP coding regions

An inverse-PCR mediated site-directed mutagenesis experiment [18] was conducted on the donor plasmid $pO^{R2/1975}$ to delete the coding region corresponding to the amino acid residues 93–143 of the NSP 3A. After the PCR, an aliquot of the reaction mixture was used to transform chemically competent *Escherichia coli* (*E. coli*) XL1 blue cells (Agilent Technologies, U.K.) and ampicillin-resistant colonies were screened for recombinant plasmid. Positive clones were characterised by nucleotide sequencing (ABI 3130 Genetic analyzer, Applied Biosystems, CA, USA). The resultant recombinant plasmid was designated as $pO^{R2/1975\text{-}\Delta\Delta3A}$. A second round of inverse-PCR mediated site-directed mutagenesis experiment was conducted on the plasmid $pO^{R2/1975\text{-}\Delta3A}$ to delete the coding region corresponding to the amino acid residues 10–37 of the NSP 3B. The final mutated genome-length cDNA clone was designated as $pO^{R2/1975\text{-}\Delta3A\text{–}\Delta3B}$. All the site-directed mutagenesis experiments were conducted using the Q5 Site-directed mutagenesis kit (NEB, USA), as per the manufacturer's instruction. The primers used for the deletion of the coding regions in the NSP 3A and 3B are listed in Table 1.

2.3. Transfection and rescue of mutant virus from the infectious cDNA clone

The plasmid $pO^{R2/1975\text{-}\Delta3A\text{–}\Delta3B}$ was linearised at the *Not*I site following the poly (A) tract and used as a template for RNA synthesis using T7 high yield RNA synthesis kit (NEB, USA), according to the manufacturer's protocol. BHK-21 cells were transfected with these synthetic RNA by chemical transfection method as described previously [19]. The transfected BHK-21 cell monolayer was washed and GMEM with 2% FBS was added, and incubated up to 48 h at 37 °C. After successive passages in BHK-21 cells, mutant virus stock was prepared and the deletion of coding sequence corresponding to the amino acid residues 93–143 of the NSP 3A and 10–37 of the NSP 3B was verified through nucleotide sequencing.

2.4. Identification and characterisation of rescued mutant virus

Rescued mutant virus was identified using the serotype specific antigen-ELISA as described earlier [20]. Affinity purified bovine polyclonal antibody specific for the truncated 3AB (t3AB) protein was purified from the bovine convalescent serum by immune-affinity chromatography (explained below) and used in Western blot, immunocyto-chemistry and immune-fluorescent assay to analyse the expression of marker antigen expression of mutant FMDV.

For Western blot assay, cell-culture supernatant of wild-type and mutated viruses (both at 10th passage) were separated by SDS-PAGE and transferred onto 0.45 μm nitro-cellulose membrane. Subsequently, the membrane was incubated with affinity purified t3AB specific bovine antiserum (1:200). Following incubation with rabbit anti-cow immunoglobulin/HRP conjugate (DAKO, Denmark), the membrane was developed with DAB substrate solution.

For immune-cytochemistry, BHK-21 cell monolayers grown on glass coverslips were infected either with wild type or mutated FMDV (both at 10th passage) at a multiplicity of infection (m.o.i) of 5. The infected cells were fixed with cold acetone:methanol (1:1) mixture for 20 min at room temperature, after 7–8 h of infection. After washing the fixed cells twice with PBS, affinity purified t3AB specific bovine antibody and rabbit anti-cow immunoglobulin/HRP

Table 1
List of Oligonucleotide primers used in this study.

Primers designation	Nucleotides sequence (5′-3′)	Nucleotide position[a]	Purpose
3A Δ93–144 F	AAACCCGTGGAGGAACAAC	5650–5668	For deletion of amino acid residues 93–144 of NSP 3A
3A Δ93–144 R	CTCATTCACTGCGTCATCC	5631–5649	For deletion of amino acid residues 93–144 of NSP 3A
3B Δ10–37 F	GTGAAAGCAAAAGCCCCGGTC	5707–5727	For deletion of amino acid residues 10–37 of NSP 3B
3B Δ10–37 R	ACGCTCAAGTGGCCCGGC	5689–5706	For deletion of amino acid residues 10–37 of NSP 3B

[a] Nucleotide position is relative to the FMDV serotype O IND R2/1975 full-genome length infection clone with deletion of amino acid residues 93–143 and 10–37 in the non-structural protein 3A and 3B, respectively (GenBank accession number KR139753).