Construction of an infectious cDNA clone of foot-and-mouth disease virus type O_1BFS 1860 and its use in the preparation of candidate vaccine

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Foot-and-mouth disease virus (FMDV) serotype O is the most predominant among the endemic serotypes in India. A stable, full-length cDNA clone of FMDV type O_1BFS 1860 preceded by a bacteriophage T7 polymerase promoter was assembled in a plasmid vector pGEM^R-7Zf(–). An ~8.2 kb PCR product was amplified from the cDNA clone and a full-length RNA was generated from it by *in vitro* transcription. Transfection of BHK-21 cells with the *in vitro* transcripts resulted in the production of infectious recombinant FMDV particles as evidenced by cytopathic effects (CPE). Further, characterization of the recombinant virus by immunofluorescence, microneutralization test (MNT), antigen ELISA, RT-PCR, plaque assay and electron microscopy revealed similarity to the parental strain. The immunogenicity of an oil-adjuvant vaccine prepared using the inactivated recombinant virus was tested in guinea pigs and cattle. Neutralizing antibodies were produced in both vaccinated guinea pigs and cattle. Vaccinated animals were protected on challenge. The results demonstrated that the recombinant virus was as stable and effective as the parental strain for the preparation of inactivated vaccine, suggesting the potential application of this strategy to make genetically engineered FMDV vaccines.

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1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating disease of cloven-hoofed animals (Grubman and Baxt 2004). FMD is caused by the foot-and-mouth disease virus (FMDV) classified under the genus *Aphthovirus* and family Picornaviridae. FMDV is a 30 nm icosahedral virus and consists of a single-stranded, positive-sense RNA genome of approximately 8500 bases surrounded by 60 copies of each of four structural proteins that form an icosahedral capsid (Grubman and Baxt 2004). The genome codes for a single, long, translational open reading frame (ORF) flanked at each end by untranslated regions (UTRs). The 5′ end of the RNA is covalently linked to a small viral

Keywords. Cattle; foot-and-mouth disease virus; full-length infectious cDNA clone; guinea pigs; *in vitro* transcribed RNA; transfection; vaccine

Abbreviations used: ABS, adult bovine serum; BEI, binary ethyleneimine; BHK-21, baby hamster kidney-21; BTY, bovine thyroid; BVS, bovine vaccinated sera; CPE, cytopathic effect; dpc, days post challenge; dpv, days post vaccination; FITC, fluorescein isothiocyanate; FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; Ig, immunoglobulin; IRES, internal ribosomal entry site; MCR, multiple cloning region; MEM, minimum essential medium; MNT, microneutralization test; MOI, multiplicity of infection; nt, nucleotide; ORF, open reading frame; PBS, phosphate buffered saline; PCR, polymerase chain reaction; p-O_1BFS, parental O_1BFS; PTA, phosphotungstic acid; RT, reverse transcriptase; UTR, untranslated region

protein (VPg) (Sangar *et al* 1977). The 5′ UTR consisting of ~1200 nucleotides (nts) is highly structured (Grubman and Baxt 2004) and contains several genetic elements necessary to control essential functions in the replication cycle (Kuhn *et al* 1990; Lopez de Quinto and Martinez-Salas 1997). The 5′ UTR of cardioviruses and aphthoviruses contains a homopolymeric poly (C) tract that separates around 150–370 bases of the 5′ end from the remainder of the genome (Clarke *et al* 1987). For FMDV, poly (C) tracts ranging from 100 to 420 residues interspersed with an occasional U residue have been reported (Harris and Brown 1976; Escarmis *et al* 1992). The ORF encodes a single polyprotein which codes for four structural proteins (VP1, VP2, VP3 and VP4) and nine non-structural proteins (L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C and 3D). The 3′ UTR of about 90 nts is present immediately downstream of the stop codon at the end of the ORF and is followed by a genetically encoded poly (A) tract (Sáiz *et al* 2001).

Antigenic plurality among the seven serotypes of FMDV (A, O, C, Asia 1, South African Territories 1, 2 and 3), continuous co-circulation of different serotypes in a given geographical region and persistence of virus in infected or vaccinated animals make the disease very difficult to control (Balamurugan *et al* 2005). FMD is controlled by 'stamping out' in the event of outbreaks in non-endemic countries and by vaccination programmes in endemic countries. Conventional FMD vaccines are produced from chemically inactivated tissue culture-propagated virus. Effective vaccination in different regions of the world requires the use of FMDV strains that are specific to the geographical locations due to the genetic and antigenic variability within different serotypes of FMD viruses (van Rensburg and Mason 2002). Expression of different FMDV antigenic structures (originating from field isolates) within the same genetic context of a tissue culture-adapted virus may give protection against multiple isolates and serve as marker vaccines.

Genetic studies of many RNA viruses were possible due to the availability of infectious cDNA clones of the entire viral genome, which served as templates for the generation of infectious RNAs (Tellier *et al* 1997). Availability of full-length infectious FMDV cDNA clones may facilitate the generation of recombinant chimeric viruses that are of epidemiological significance and possess a broader antigenic spectrum by making specific substitutions. Also, such full-length FMDV cDNA clones are helpful in understanding virus replication, pathogenicity and vaccine development (van Rensburg and Mason 2002; Boyer and Haenni 2002). There are reports on the generation of full-length infectious cDNA clones (Zibert *et al* 1990; Reider *et al* 1993; Liu *et al* 2004) and chimeric viruses of FMDV (Reider *et al* 1993; Mason *et al* 1997; Almeida *et al* 1998; Sáiz *et al* 2001; van Rensburg and Mason 2002; Fowler *et al* 2008). Development of many more such cDNA clones is likely to aid in quick and easy development of chimeric viruses, genetically engineered attenuated vaccines, and recombinant viral vector vaccines for effective FMDV control.

Among the three serotypes that are circulating in India, serotype O causes around 80% of the outbreaks. Type A and Asia1 cause the remaining outbreaks. In this study, we report the design and construction of a full-length cDNA clone of FMDV type O_1BFS 1860 as a model system, transfection of baby hamster kidney-21 (BHK-21) cells with *in vitro* transcribed RNA derived from full-length cDNA and recovery of infectious recombinant viruses. Further, the growth characteristics of recombinant virus and the ability of an inactivated recombinant virus vaccine to stimulate an immune response in guinea pigs and cattle were studied.

2. Materials and methods

2.1 *Cell lines and viruses*

A BHK-21 cell line maintained by the Cell Culture Laboratory, Indian Immunologicals Limited (IIL), India was grown in minimum essential medium (MEM) (Invitrogen, USA) supplemented with 10% adult bovine serum (ABS) (Selbourne, UK). Primary bovine thyroid (BTY) cells were isolated from the thyroid glands of 1-day-old calves and were grown in MEM supplemented with 10% ABS. Tissue culture-adapted O_1BFS 1860 available with the virus seed laboratory, Quality Control Department, IIL was used to generate the cDNA clone and was referred to as parental O_1BFS (p-O_1BFS) throughout the study.

2.2 *Animals*

Guinea pigs weighing about 250–300 g were used for immunization experiments. Naïve, unvaccinated, crossbred male cattle calves aged 11–12 months free of FMDV antibodies were used for studying the immunogenicity and efficacy of the recombinant virus vaccine.

2.3 *Construction of FMDV type O_1BFS 1860 full-length cDNA clone*

Restriction enzyme sites *Nde*1, *Nhe*1, *Not*1 and *Spe*1 were substituted in the multiple cloning region (MCR) of the vector pGEM^R-7Zf(-) (Promega, USA) by four rounds of site-directed mutagenesis using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, USA) (figure 1A) for the assembly of the full-length cDNA clone. After each round, incorporation of the restriction site was confirmed by DNA sequencing and the modified vector was used as the template for the next round of site-directed mutagenesis.

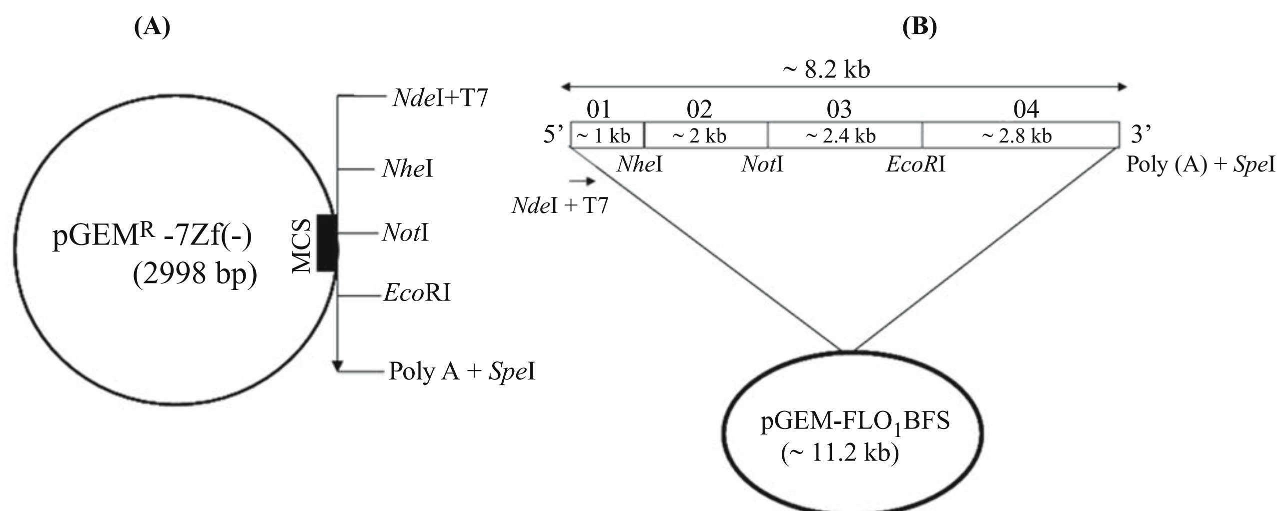

Figure 1. Construction of FMDV O_1BFS 1860 cDNA clone. (**A**) Schematic representation of modified $pGEM^R$-7Zf(–) vector showing restriction sites substituted at the multiple cloning region (MCR) by site-directed mutagenesis for the generation of full-length cDNA of FMDV O_1BFS 1860. (**B**) Schematic representation of four fragments of the complete genome of FMDV O_1BFS 1860, which were aligned in modified $pGEM^R$-7Zf(–) to generate pGEM-FLO_1BFS. The sizes of the fragments are depicted along with the flanking restriction sites. The *Nhe*I site is represented in italics which was introduced as a silent mutation at the 3′ end of fragment 1 (O1) for cloning as well as for marker analysis. pGEM-FLO_1BFS was preceded with a T7 polymerase promoter at the 5′ end and poly A bases at the 3′ end.

Table 1. List of primers with sequence information used for the construction of the full-length cDNA clone of FMDV O_1BFS 1860. Nucleotide position corresponds to the nucleotide sequence of the FMDV O_1BFS (GenBank Accession No: AY593815). Restriction endonuclease and T7 promoter sequences introduced in the primers are in bold and underlined.

Primer	Nucleotide sequence*	Nucleotide position
O1F	5′ GTC**<u>CATATG</u> NdeI <u>TAATACGACTCACTAGTGG</u> T7promoter** TTGAAAGGGGGCGCTAGGGTC 3′	1–21
O1R	5′ ACGT**<u>GCTAGC</u> NheI** TTGTTACCTCGGGGTACCTGAAGGGCATCCTTAG 3′	941–980
O2F	5′ ACGT**<u>GCTAGC</u> NheI** CTCGGGATCTGAGAAGGGGACTGGGACTTC 3′	980–1010
O2R	5′ TTG**<u>GCGGCCGC</u> NotI** CTCAGGTGTCTTGGGCGGCTCCATGCCCGGTGGGGC 3′	2981–3024
O3F	5′ TTG**<u>GCGGCCGC</u> NotI** GCACTGCATTCATGCTGAATGGGACACTGGGTTGAA 3′	3017–3060
O3R	5′ TGC**<u>GAATTC</u> EcoRI** AATTGCTGCCTCATGTTGGCCCTTCTCAATGAGAAAG 3′	5401–5443
O4F	5′ TGC**<u>GAATTC</u> EcoRI** TTTGAGGGCATGGTCCACGACTCTATTAAAGAGGAAC 3′	5438–5480
O4R	5′ GAC**<u>ACTAGT</u> SpeI** TTTTTTTTTTTTTTTTGATTAAGGAAGCGGGAAAAGCCCTTTCGG 3′	8151–8195
N1F	5′ CCTCCTTGGTAACAAGGACCCAC 3′	801–823
N1R	5′ CTGTGGTGCGTGGTAGGAAAAG 3′	1151–1172

The *Eco*RI site, which was originally present in the vector, was utilized for cloning without any modification.

RNA was extracted from the purified p-O_1BFS suspension using trizol reagent (Invitrogen, USA) and used immediately for cDNA synthesis. All the primers with restriction sites (table 1) were designed based on the published O_1BFS sequence (GenBank Accession No: AY593815). FMDV viral RNA-specific primers and Thermoscript™ Reverse Transcriptase (Invitrogen, USA) were used for first-strand cDNA synthesis, followed by polymerase chain reaction (PCR) amplification with Pfu Turbo DNA polymerase (Stratagene, USA). All the above procedures were performed as per the manufacturer's instructions. A total of four fragments covering the complete genome of p-O_1BFS were amplified with appropriate restriction enzyme sites as shown in figure 1B.

Fragment 1 (O1; ~1 kb; nucleotide [nt] 1 to 980 nts) was amplified with the *Nde*I and *Nhe*I sites at the 5′ and 3′ ends, respectively, using the O1F and O1R primers. The O1F primer also contained a promoter for the T7 RNA polymerase downstream of the *Nde*I site, and the *Nhe*I site was introduced as a silent mutation at the 3′ end of fragment

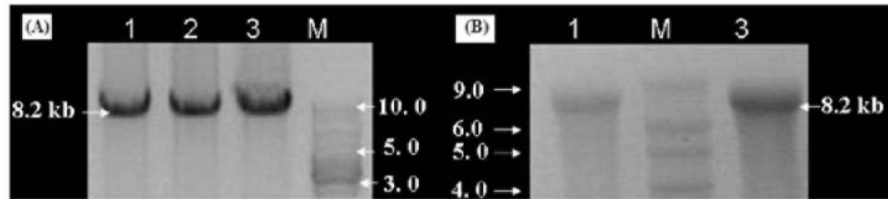

Figure 2. **(A)** Amplification of the full-length 8.2 kb PCR products (lanes 1, 2 and 3) using primers O1F and O4R from different pGEM-FLO_1BFS clones. Lane M represents the MBI Fermentas 1 kb ladder. **(B)** Glyoxal–formamide gel showing the *in vitro* transcribed RNA. Lanes 1 and 3 represent 0.5 μg and 1 μg, respectively. Lane M represents the RNA ladder (Ambion, USA).

1 (representing positions 977 G to T, 979 C to G and 980 A to C) (figure 2). In the same way, amplification was performed of fragment 2 (O2; ~2 kb; 980 nts to 3024 nts) with the *Nhe*1 and *Not*1 sites at the 5′ and 3′ ends, respectively, using the primers O2F and O2R; fragment 3 (O3; ~2.4 kb; 3024 nts to 5443 nts) with the *Not*I and *Eco*RI sites at the 5′ and 3′ ends, respectively, using the primers O3F and O3R; and fragment 4 (O4; ~2.8 kb; 5443 nts to 8195 nts) with the *Eco*RI and *Spe*1 sites at the 5′ and 3′ ends, respectively, using the primers O4F and O4R (figure 2). The PCR fragments were gel purified (Qiagen, Germany) and cloned into the pCR^{R}2.1-TOPO^R vector (Invitrogen, USA). The authenticity of each fragment was confirmed by sequencing.

To generate a full-length cDNA clone, the four cDNA fragments (O1, O2, O3 and O4) released from the respective TOPO vectors were moved sequentially into the modified pGEM^R-7Zf[–] vector (figure 1B). After each round of cloning, alignment of the fragment was checked by DNA sequencing and an authentic plasmid was used as a vector for ligation of the next fragment. The final clone harbouring the full-length cDNA was confirmed by sequencing and named pGEM-FLO_1BFS.

2.4 *RNA synthesis* in vitro

pGEM-FLO_1BFS was used as a template for the amplification of an 8.2 kb PCR product using primers O1F and O4R (figure 2A). This PCR product was purified (Qiagen, Germany) and checked by internal restriction digestions and sequencing. This was used as a template for *in vitro* transcription using the MEGAScript^{TM} T7 kit (Ambion, USA) according to the manufacturer's instructions. Full-length transcripts were precipitated with lithium chloride to remove free nucleotides, denatured with glyoxal and electrophoresed in 1% agarose gels as per Maniatis *et al* (1982). The RNA was stained with toluidine blue and quantified by spectroscopy.

2.5 *Transfection and recovery of recombinant virus*

Two millilitre of BHK-21 cells (2 × 10^5 cells/well) were seeded in 6-well plates (Nunc, USA). The cells were grown overnight at 37ºC in the presence of 5% CO_2 to 80% confluence. The cells were then transfected with 5 μg of *in vitro* transcribed RNA mixed with lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. BHK-21 cells transfected with lipofectamine alone and RNA extracted from p-O_1BFS with the RNeasy kit (Qiagen, Germany) mixed with lipofectamine were used as the negative and positive controls, respectively. After 2 h of exposure to lipofectamine and RNA, the monolayer was washed with virus maintenance medium; fresh medium was added and incubated at 37ºC with 5% CO_2 for 48 h. The cells were examined for CPE at 24 and 48 h. After 48 h, the viral harvests were passaged three times in a BHK-21 monolayer after a freeze and thaw cycle each time. Recombinant virus (r-O_1BFS) derived from the pGEM-FLO_1BFS and p-O_1BFS harvests thus obtained were stored at –70ºC until further use.

2.5.1 *Inactivation and purification of r-O_1BFS:* BHK-21 cells maintained as described above were infected with r-O_1BFS and p-O_1BFS with a multiplicity of infection (MOI) of 1.0 and incubated at 37ºC for 48 h. The cell culture fluid was clarified and the cell lysate was inactivated with two doses of binary ethyleneimine (BEI) (Bahnemann 1975). Inactivation kinetics and virus amplification tests were conducted to ensure absence of live virus in the suspension. Inactivated virus antigens were concentrated by ultrafiltration and used for vaccine formulations.

The inactivated FMDV antigen with a sedimentation coefficient of 146S (146S antigen) was purified by ultracentrifugation as described by Doel *et al* (1982). Briefly, 50% v/v saturated ammonium sulphate (4 M) solution was added to the clarified supernatant cultures and kept at 4ºC overnight. The suspension was centrifuged at 2000 rpm for 30 min at 4ºC. The precipitate was resuspended in 0.04 M phosphate buffer and centrifuged at 10 000 rpm for 60 min at 4ºC. The resultant supernatant was again centrifuged at 285 000 *g* using an ultracentrifuge (Beckman, USA) for 2 h. The pellet was carefully dissolved in 0.04 M phosphate buffer and the antigen-containing solution was overlaid on a 20–40% w/v linear gradient of sucrose and centrifuged in a swing-out rotor (Beckman, USA) at 40 000 rpm for 2 h at 4ºC. The peak virus-containing fraction was collected using a density gradient fractionator (Isco, USA) and quantified at

254 nm using a UV visible spectrophotometer (Beckman, USA) (Doel and Mowat 1985). The purified 146S antigen was stored in liquid nitrogen until further use.

2.6 *Characterization of r-O_1BFS*

2.6.1 *Immunofluorescence microscopy:* BHK-21 monolayer cells ($15x10^5$/ml) were infected with r-O_1BFS and p-O_1BFS. Mock-inoculated cells with phosphate buffered saline (PBS) were used as the negative control. The monolayer was fixed at different time intervals of 4 h, 8 h, 12 h and 16 h post infection with 50% acetone for 30 min. The monolayer was washed thrice with PBS, Type O-specific monoclonal antibody in PBS containing 10% foetal bovine serum (Moregate, USA) and 0.005% Tween-20 (PBS–FBS–T) (Sigma, USA), and added to the fixed monolayer. The plates were incubated at 37°C for 1 h. The buffer was carefully removed and the monolayer washed 5 times with PBS containing 0.005% Tween-20 (PBS–T). Anti-mouse immunoglobulin (Ig)G conjugated with fluorescein isothiocyanate (FITC) in PBS–FBS–T (1:2000 dilution) was added to the monolayer. The plates were incubated and washed as described above and finally once with PBS alone. The presence or absence of intracellular cytoplasmic fluorescence was observed and captured using a fluorescent microscope (Zesis, Japan) (Timm *et al* 1983).

2.6.2 *Micro neutralization test:* Two-dimensional micro neutralization test (MNT) was performed in triplicate, using r-O_1BFS and p-O_1BFS against bovine vaccinated sera (BVS) raised against p-O_1BFS. The results were compared for significance using the *t*-test (Rweyemamu and Hingley 1984).

2.6.3 *RT-PCR analysis:* The presence of FMDV in the viral harvests was confirmed by reverse transcriptase (RT)-PCR using serotype-specific primers (Knowles and Samuel 1994).

2.6.4 *Virus typing and genetic marker analysis:* FMDV typing by antigen detection ELISA was performed to detect the presence of FMDV and identification of the serotype as per the procedure described by Hamblin *et al* (1984) using polyclonal rabbit and guinea pig sera raised against FMDV O, A and Asia1 serotypes. The restriction site *Nhe*I was engineered into the cDNA clone (figure 1B) to distinguish between r-O_1BFS and p-O_1BFS. RNA from r-O_1BFS was isolated with the RNeasy mini kit (Qiagen, Germany), and p-O_1BFS harvested from 24 h post-transfected supernatants and subjected to c-DNA amplification and PCR as mentioned in Section 2.3 using the primers N1F and N1R. The 350 nt fragment was cloned into the pCR^{R}2.1-TOPO^R vector (Invitrogen, USA). Presence of the *Nhe*I site was determined by sequencing and restriction digestion using a combination of *Nhe*I and *Eco*RI (*Eco*RI was present on either side of the insert in the vector).

2.6.5 *Virus titration and serology:* Plaque assay on BHK-21 was used to determine the titre of r-O_1BFS (Bachrach *et al* 1957). Sera were tested for the presence of neutralizing antibodies against FMDV by the virus neutralization test (Golding *et al* 1976) and antigen ELISA (Hamblin *et al* 1984)

Virus neutralization tests were performed in flat-bottomed tissue culture-grade microtitre plates (Nunc, USA) using the method previously described (Golding *et al* 1976). Antibody titres were expressed as the reciprocal of the final dilution of serum in the serum/virus mixture, which neutralized an estimated 100 $TCID_{50}$ of virus at the 50% end-point estimated according to the method of Karber (1931). Serum samples were assayed for the presence of antibodies against non-structural FMDV poly protein 3ABC using a commercial kit (Ceditest FMDV-NS, Cedi-diagnostics, The Netherlands) and an in-house indirect ELISA using Ph-3AB antigen (Hema *et al* 2007).

2.6.6 *Electron microscopy:* The purified r-O_1BFS (200 μg/ml) was applied onto carbon-shadowed, formvar-coated grids and visualized by negative staining with 1% phosphotungstic acid (PTA), pH 7.0 (Sigma, USA). These grids were viewed under a high-resolution transmission electron microscope (Hitachi H 7500, Japan) at a magnification of 40 000x.

2.7 *Preparation of oil adjuvant vaccine:* Monovalent vaccines containing 10 μg of inactivated r-O_1BFS and p-O_1BFS antigens were prepared using Montanide Oil ISA 206 (Sepic, France). The preparations were checked for sterility, innocuity and stored at 4ºC until further use.

2.8 *Animal studies*

2.8.1 *Immunogenicity studies in guinea pigs and cattle:* Five groups of 10 guinea pigs each were taken. Groups 1 and 2 were inoculated with p-O_1BFS 1860 monovalent vaccine. Groups 3 and 4 were inoculated with r-O_1BFS monovalent vaccine. Group 5 was used as an unvaccinated control. Groups 1 and 3 were boosted with p-O_1BFS vaccine, while groups 2 and 4 were boosted with r-O_1BFS vaccine on day 21. Blood was collected on 0, 21 and 35 days post vaccination (dpv).

Two groups of 10 cattle calves each were inoculated with a p-O_1BFS vaccine and r-O_1BFS vaccine, respectively. Five cattle calves were used as unvaccinated controls. Blood was collected on days 0 and 21 post vaccination. Serum was separated, inactivated at 56ºC and stored at –20ºC until further use.

2.8.2 *Efficacy studies in cattle:* Two groups of 4 naïve cattle calves (primo vaccinates) each were inoculated with 2 ml of p-O_1BFS vaccine and r-O_1BFS vaccine, respectively. Four cattle calves were used as unvaccinated controls. Blood was collected on 0 and 21 dpv, the serum separated, inactivated

and stored as described above until further use. The animals were challenged by the intradermolingual route on 28 dpv using 10^4 ID_{50} of homologous cattle challenge virus. The animals were observed for 10 days post challenge (dpc) for the development of clinical disease. Serum, plasma, probang samples and nasal secretions were collected on days 1–7 and 10 dpc, and the serum and plasma samples stored at –20ºC while the probang samples and nasal secretions were stored at –70ºC until further use.

2.8.2.1 *Virus isolation and identification:* Epithelial tissue, nasal secretions and probang samples from FMDV-infected animals were examined for the presence of virus by inoculation of a monolayer of BTY cells (Snowdon 1966). Three BTY tubes were used for each sample (100 μl of sample/tube). Inoculated tubes were incubated at 37ºC and examined at 24, 48 and 72 h for CPE. Presence of virus was confirmed by antigen ELISA (Hamblin *et al* 1984) and RT-PCR (Knowles and Samuel 1994)

2.8.2.2 *Real-time quantitative RT-PCR assay:* The amount of viral RNA in plasma, nasal secretions and probang samples was quantified by real-time RT-PCR (Shaw *et al* 2007). For the generation of standard curves, an FMDV RNA standard was synthesized *in vitro* from a plasmid containing a 79 bp insert of the internal ribosomal entry site (IRES) of a type O FMDV (kindly provided by Dr Donald King, Institute for Animal Health, UK) using a MEGAScript™ T7 kit (Ambion, USA) and analysed by spectroscopy. Samples with a cycle threshold (Ct) value of 35 or less were considered positive (Fowler *et al* 2008).

3. Results

3.1 *Assembly of full-length cDNA clone*

The four cDNA fragments representing the entire full-length RNA genome of FMDV p-O_1BFS were amplified and cloned into TOPO vectors (pTOPO-O1, pTOPO-O2, pTOPO-O3 and pTOPO-O4). These constructs were digested to release the four DNA fragments with appropriate

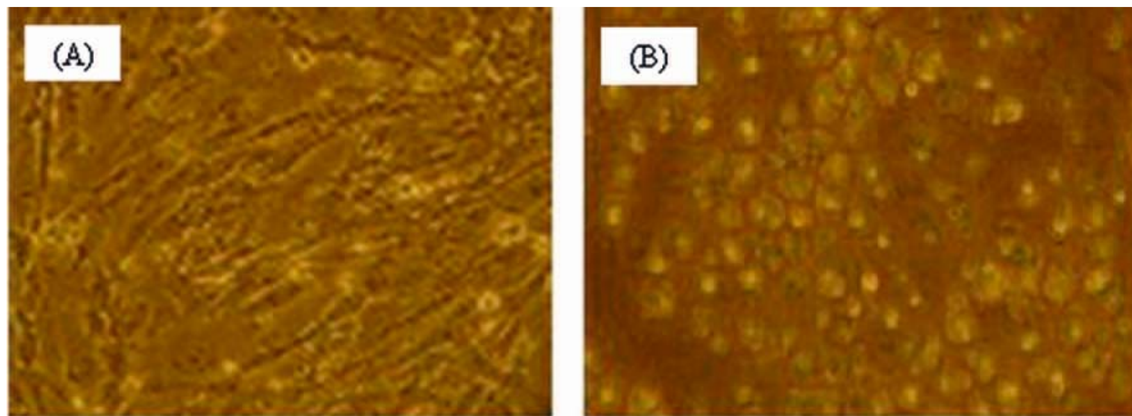

Figure 3. Analysis of lipofectamine + *in vitro* RNA transfected BHK-21 cells.(**A**) Mock-inoculated BHK-21 cells. (**B**) Cytopathic effect (CPE) caused by the transcripts derived from pGEM-FLO_1BFS.

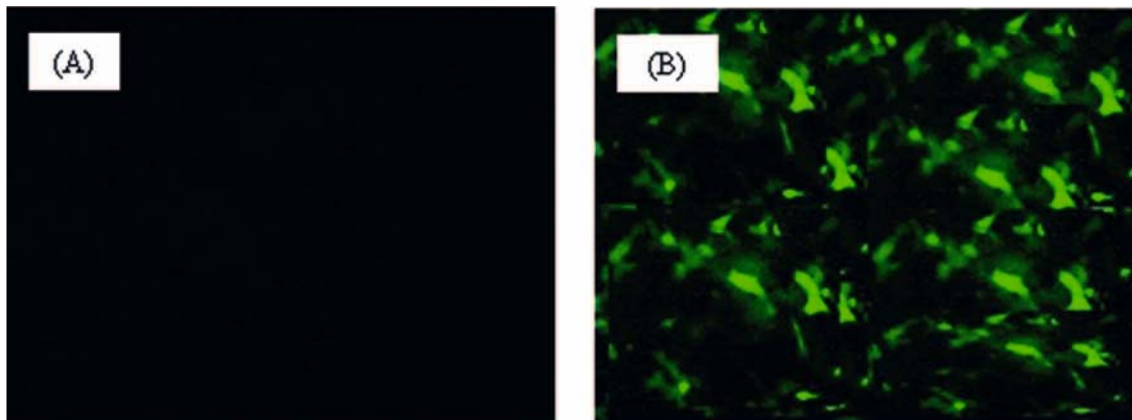

Figure 4. Immunofluorescence analysis of recombinant virus (r-O_1BFS) in BHK-21 cells stained with an FMDV serotype O-specific monoclonal antibody and anti-mouse IgG conjugated with FITC. (**A**) Mock-inoculated BHK-21 cells (**B**) r-O_1BFS-infected BHK-21 cells.

restriction sites and sequentially aligned into a modified pGEM[R]-7Zf(–) vector using a construction strategy (figure 1A, B). Nucleotide sequencing indicated ≥99 homology to the p-O_1BFS sequence with a 5′ poly $[C]_{12}$ tract and a 3′ poly $[A]_{16}$ tract. Using pGEM-FLO_1BFS as template, an ~8.2 kb product preceded by the T7 polymerase promoter was amplified (figure 2A).

Table 2. Results of antigen ELISA performed for the r-O_1BFS and p-O_1BFS harvests

Sample	Neat	Neat/2	Neat/4	Antigen blank
r-O_1BFS Transfection harvest	0.726	0.677	0.624	0.004
r-O_1BFS Passage 3	0.529	0.499	0.471	0.004
p-O_1BFS Positive control	0.581	0.514	0.447	0.002
A IND 17/82	0.006	0.008	0.004	0.005
Asia1 IND 63/72	0.009	0.008	0.002	0.006
Negative control	0.005	0.008	0.002	0.005

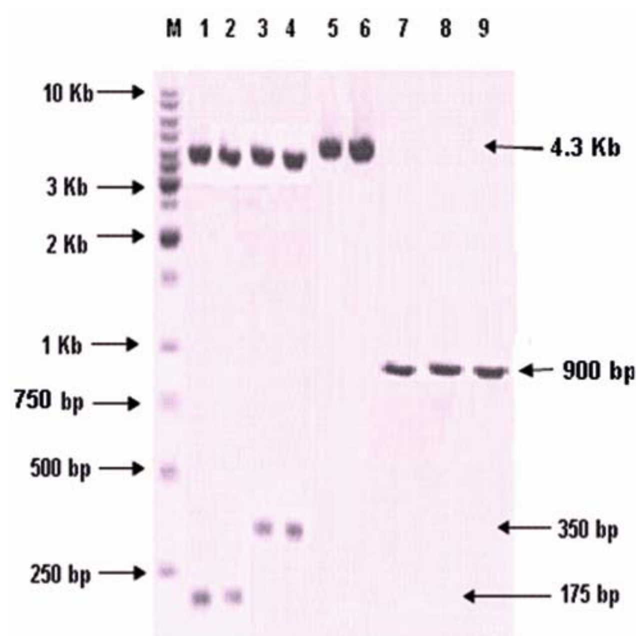

Figure 5. Agarose gel electrophoresis of RE digestion and RT-PCR. Lane M, 1 kb DNA ladder. Lanes 1 and 2, *Eco*RI and *Nhe*I RE digested pTOPO-R showing release of a 175 bp fragment indicating the presence of an Nhe1 site. Lanes 3 and 4, *Eco*RI and *Nhe*I RE digested pTOPO-P showing release of a 350 bp fragment indicating the absence of an *Nhe*I site. Lanes 5 and 6, uncut pTOPO-R and pTOPO-P. Lanes 7 and 8, RT-PCR product specific for type O (900 bp) obtained using RNA isolated from r-O_1BFS transfection harvest and r-O_1BFS Passage 3. Lane 9, RT-PCR product specific for type O obtained from RNA isolated from p-O_1BFS.

3.2 In vitro *transcription and transfection of BHK-21 cells with RNA*

An ~8.2 kb RNA was transcribed from the full-length PCR product by T7 polymerase as shown in the glyoxal–formamide agarose gel (figure 2B). For the recovery of infectious recombinant virus, BHK-21 was transfected with the *in vitro* transcribed RNA using lipofectamine 2000. Apparent CPE was observed after 48 h of incubation (figure 3B), which was indistinguishable from the effect produced by transfected p-O_1BFS RNA, indicating the infectious nature of the *in vitro* transcribed RNA. Recombinant virus (r-O_1BFS) was recovered from the cell lysates, inactivated, purified and used for further characterization.

3.3 *Characterization of the recovered recombinant virus*

3.3.1 *Immunofluorescence microscopy:* The presence of r-O_1BFS was established by immunofluorescence testing using FITC-labelled secondary antibody. Intracellular cytoplasmic fluorescence was observed from 12 h onwards in r-O_1BFS (figure 4B) and p-O_1BFS infected plates (results not shown), which indicated the presence of viral proteins. Fluorescence was not observed in the mock-inoculated plates (figure 4A).

3.3.2 *Microneutralization assay:* Microneutralization assay performed in triplicate confirmed that the progeny virus r-O_1BFS was homologous to the parent virus with *r* values of 0.91–1.12 and a *P* value >0.05.

3.3.3 *Typing and genetic marker analysis:* For typing of r-O_1BFS, the lysates obtained 48 h post transfection with *in vitro* transcribed RNA, p-O_1BFS RNA (positive control) and buffer (negative control) were passaged thrice on BHK-21 cells and the viruses typed at each passage using antigen ELISA and RT-PCR. The recombinant virus was typed as 'O', similar to the p-O_1BFS in antigen ELISA (table 2). Typing of recombinant virus was further confirmed by the amplification of a type 'O'-specific product of 900 bp and its sequencing (figure 5).

A ~350 bp product containing the *Nhe*1 site as genetic marker was amplified by two-step RT-PCR from RNA extracted from r-O_1BFS and p-O_1BFS. The PCR products were cloned into the pCR[R]2.1-TOPO[R] vector and named pTOPO-R and pTOPO-P, respectively. Digestion of pTOPO-R and pTOPO-P with *Nhe*I and *Eco*RI resulted in the release of fragments of ~175 bp and ~350 bp, respectively, indicating the presence of the recombinant virus, which was also confirmed by sequencing.

3.3.4 *Virus titration:* Plaques produced by the recombinant virus were similar to those produced by p-O_1BFS (figure 6B, C). The titre of recombinant virus r-O_1BFS was 6.62 pfu/ml at the end of three passages in BHK-21.

3.3.5 *Electron microscopy:* Electron microscopic study revealed the presence of spherical particles about 30 nm in diameter indicating that the virus recovered from the transfected cells was indeed FMDV (figure 7).

3.4 *Neutralizing antibody response in guinea pigs and cattle:* In order to determine whether r-O_1BFS induced a specific antibody response against p-O_1BFS, inactivated vaccines were prepared incorporating r-O_1BFS and p-O_1BFS. Both the inactivated vaccines were used for vaccinating guinea pigs and cattle. The mean antibody titres in guinea pigs of groups 1 and 2 vaccinated with r-O_1BFS, and groups 3 and 4 vaccinated with p-O_1BFS were $10^{1.48\pm0.20}$ and $10^{1.35\pm0.23}$, respectively, at 21 dpv. Subsequent to the booster on day 21, an anamnestic response was noticed in all the four vaccinated groups and the antibody titres ranged from $10^{1.77\pm0.11}$ to $10^{1.95\pm0.13}$ (figure 8A). The mean antibody titres in cattle on 21 dpv were $10^{1.96\pm0.30}$ and $10^{1.71\pm0.32}$, respectively, for the p-O_1BFS vaccine group and r-O_1BFS, respectively (figure 8B) for cattle.

3.5 *Challenge test results*

Four crossbred cattle calves vaccinated with p-O_1BFS and r-O_1BFS were challenged on 28 dpv, by intradermolingual inoculation of virulent cattle challenge virus at 10^4 cattle ID_{50} in two sites. On the day of challenge, the log antibody titre for the two groups was $10^{2.11\pm0.39}$ and $10^{1.77\pm0.28}$, respectively (figure 9A). All the animals vaccinated with p-O_1BFS and r-O_1BFS vaccines were completely protected (100%) against virulent challenge. The control animals showed pyrexia from 2 to 4 dpc while the vaccinated animals did not show any rise in body temperature (figure 9B). All the four unvaccinated control animals showed generalized lesions with vesicles in all four feet. Virus could be isolated from the nasal secretions on the 4th and 5th dpc in two animals in the r-O_1BFS group and on the 3rd, 4th and 5th dpc from the control animals (table 3). Virus could not be isolated from the plasma samples but could be isolated from the probang samples at 8 and 10 dpc in control animals and from one of the r-O_1BFS vaccinated groups at 10 dpc. Virus replication was evident in the plasma of all the control animals (figure 10A) while virus excretion from nasal secretions (figure 10B**)** and probang samples (figure 10C) were similar in both the groups. However, the amount of virus load was significantly lower in the vaccinated cattle when compared with the unvaccinated control group. At 10 dpc, one animal in each vaccinated group and all the animals in the control group showed anti-FMDV–NSP antibodies in the serum.

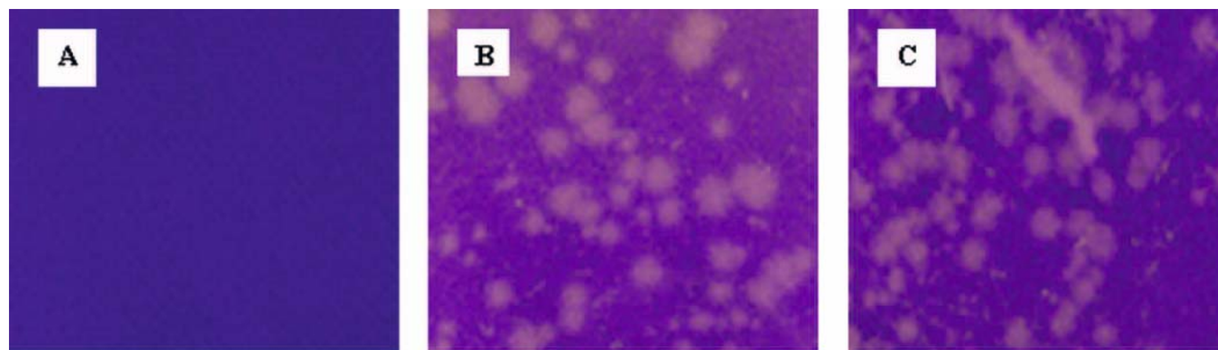

Figure 6. Plaque assay of r-O_1BFS and p-O_1BFS on BHK-21 cells. (**A**) Cell control. (**B**) Plaques produced by r-O_1BFS. (**C**) Plaques produced by p-O_1BFS.

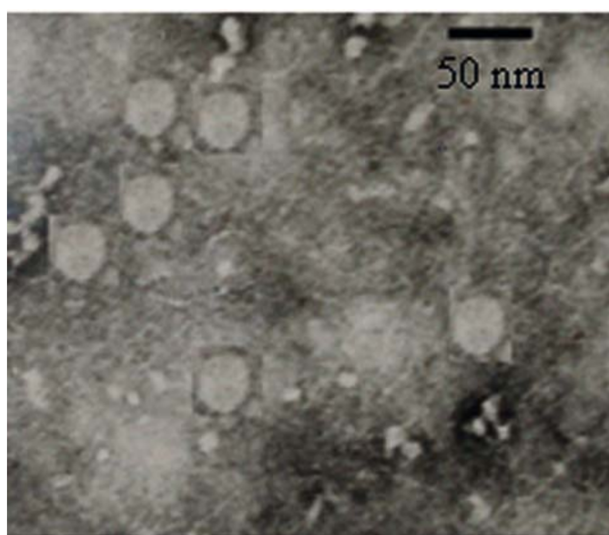

Figure 7. Transmission electron micrograph of negatively stained r-O_1BFS particles. (bar represents 50 nm)

4. Discussion

We describe the construction of a full-length cDNA clone of tissue culture-adapted FMDV type O_1BFS 1860. RNA derived *in vitro* from this cDNA proved to be infectious in BHK-21 cells.

An inherent problem with RT-PCR is the appearance of mutations, which was circumvented by using a proofreading

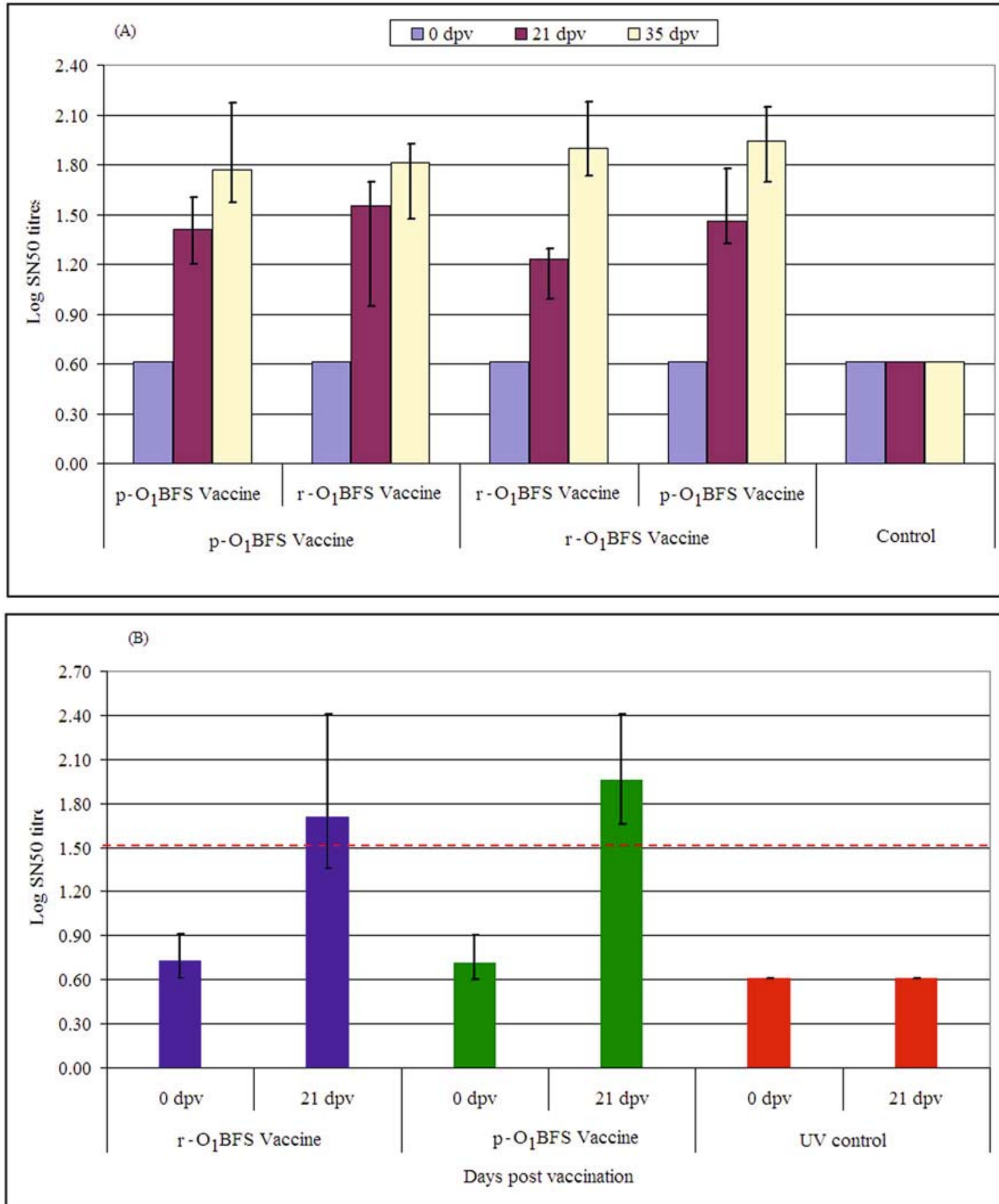

Figure 8. Immune response to p-O_1BFS 1860 and r-O_1BFS vaccines with and without booster (**A**) in guinea pigs and (**B**) in cattle.

thermostable DNA polymerase in our study and sequencing individual cDNA fragments before final assembly. In order to eliminate any possibility of the presence of extra nucleotides hindering the process of transcription/generation of recombinant virus as a result of the cloning strategy, pGEM-FLO_1BFS was subjected to PCR and the resultant full-length PCR product was used for *in vitro* RNA transcription.

Sequence analysis indicated that the full-length cDNA clone of FMDV type O_1BFS contained a poly [C] tract of 12 nucleotides and a recombinant progeny virus was recovered from the transfected cells. The precise role of the poly [C] tract in the life cycle of cardio-aphthoviruses is not known (Zibert *et al* 1990). For FMDV, descriptive studies have suggested that changes in the length of the poly [C]

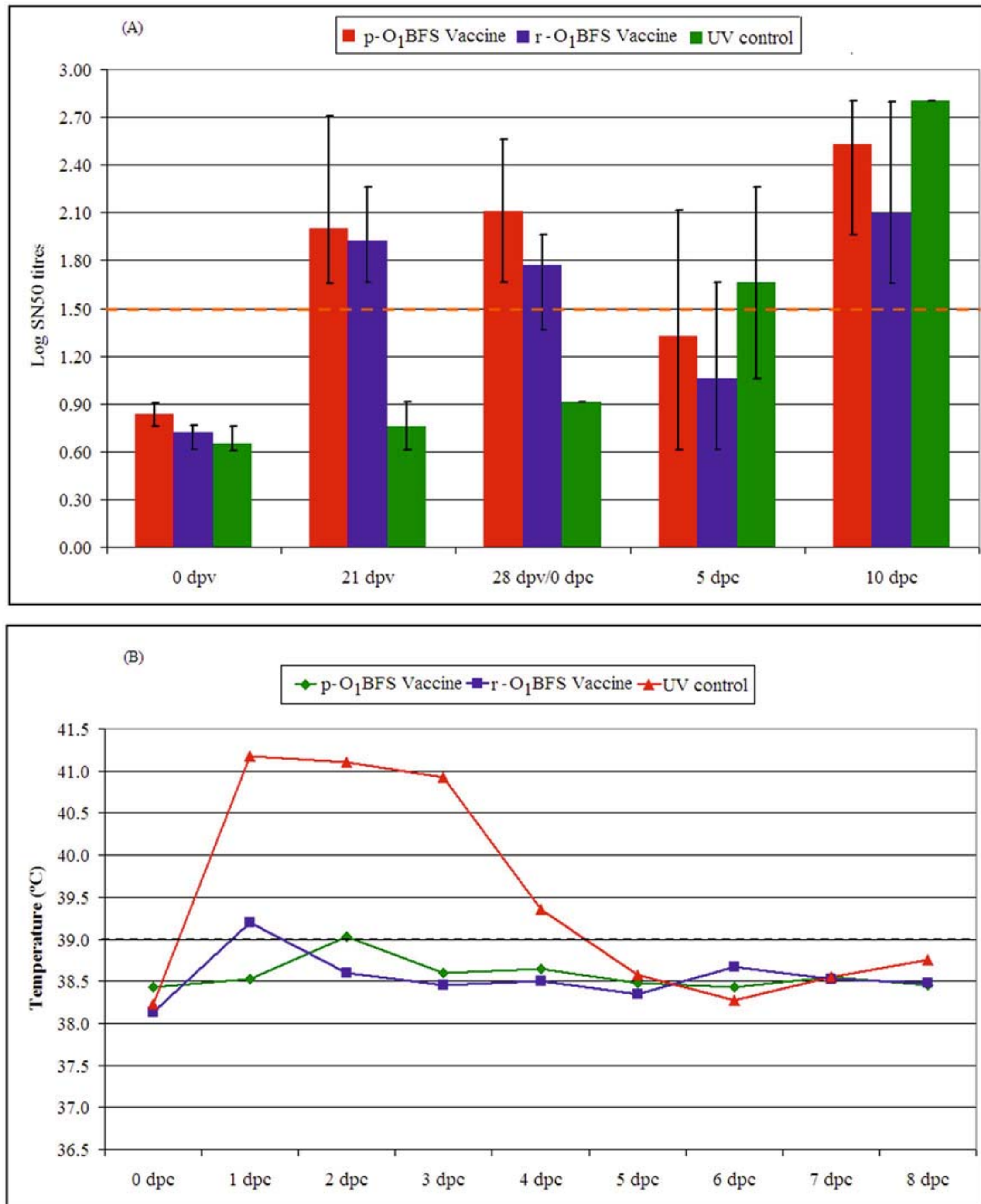

Figure 9. (**A**) Mean antibody titres of cattle vaccinated with p-O_1BFS and r-O_1BFS vaccine and challenged with virulent homologous cattle challenge virus. (**B**) Mean rectal temperature (ºC) of cattle following challenge with O_1BFS virus.

tract may be associated with changes in virulence, but these studies have been unable to relate poly [C] tract length to any biological function of the virus, either in tissue culture or *in vivo* (Harris and Brown 1977). Zibert *et al* (1990) considered that FMDV had an absolute requirement for a poly [C] tract in order to replicate in cell culture. However, Rieder *et al* (1993) successfully constructed infectious cDNA clones of FMDV type A12, which contained poly [C] tracts of 2–35 nucleotides; all of the recovered viruses were equally virulent in mice indicating that poly [C] tract length had no effect on virulence in this animal model. Also, in a large-scale survey of FMDV in South America (Cao *et al* 1991),

strains of the virus with very short poly[C] tracts but with unaltered pathogenicity for cattle were observed. Multiple attempts to commercially synthesize a DNA construct with 32 Cs with the idea of swapping it into the full-length backbone were not successful (data not shown).

The poly [A] tract of the FMDV virus RNA has been reported to be essential for infectivity of the RNA (Liu *et al* 2004). This infectivity was found to increase with poly [A] length (Baxt *et al* 1979). However, the minimum size of the poly [A] tract required for infectivity of the FMDV RNA is unknown (Liu *et al* 2004). We introduced 16 adenosine residues at the 3′ end of the full-length cDNA clone. Viral RNA from type A FMDV, which contains less that 10 A residues at the 3′ end, has been reported to have the same infectivity as viral RNA that contained approximately 40 residues of adenosine at the 3′ end (Grubman *et al* 1979). Our results demonstrate that RNA derived from an FMDV cDNA clone containing a 16 bp long poly [A] tail has similar infectivity as the p-O_1BFS.

To exclude the possibility that the virus recovered from the transfected cells was due to contamination of the sample with wild-type virus, an *Nhe*I site was engineered as a part of the cloning strategy into the full-length clone, which served as a genetic marker. Restriction digestion of pTOPO-R and

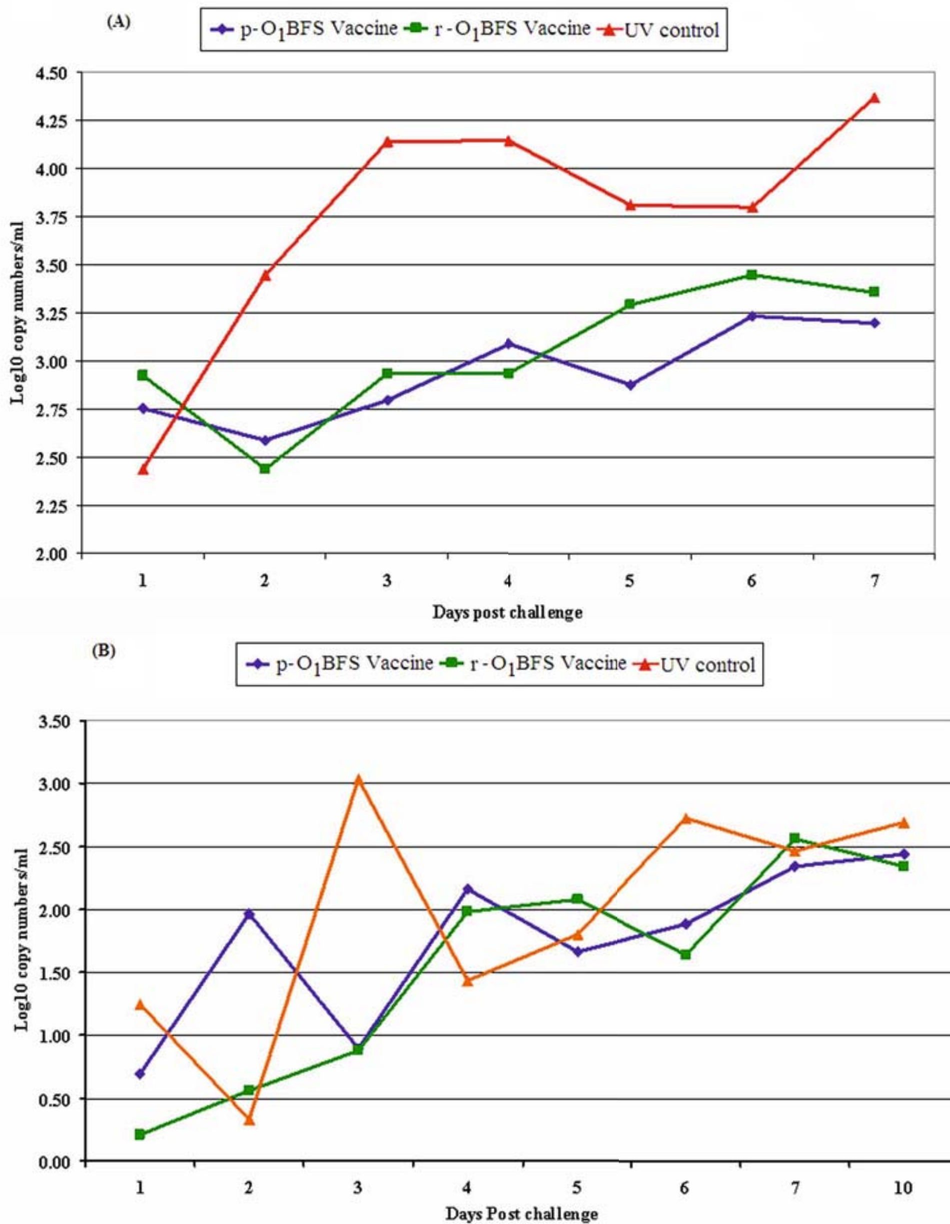

Figure 10A,B. For caption. *see* page No. 56.

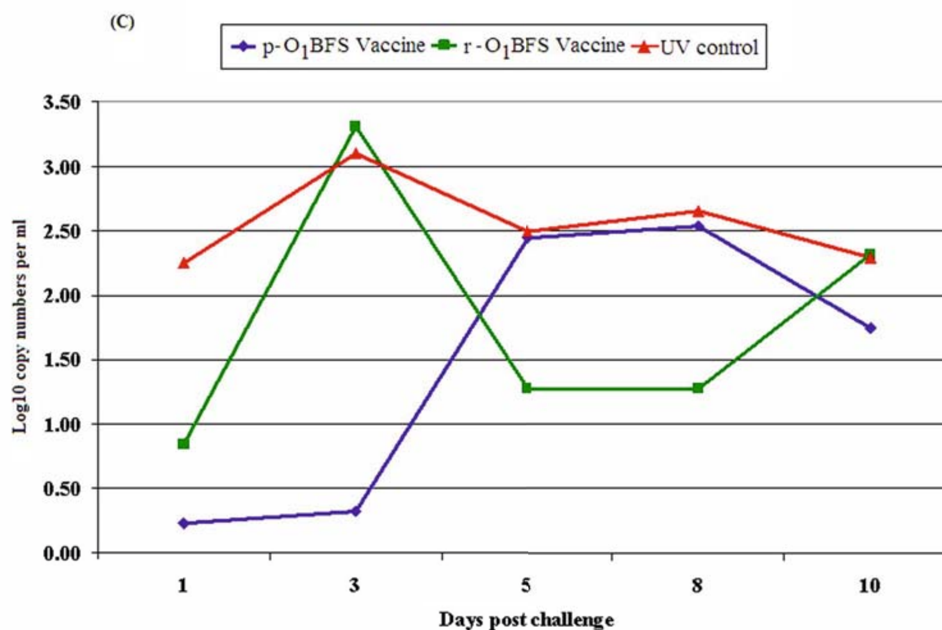

Figure 10. Mean FMDV copy number detected by qRT-PCR; (**A**) from plasma, (**B**) from nasal secretions and (**C**) from probang samples in challenged crossbred cattle.

Table 3. Virus isolation and RT-PCR results for nasal, plasma and oesophagopharyngeal samples taken between 0 and 10 days post challenge (dpc).

Animal ID	Nasal secretion	Plasma	Probang
C-2070	-	-	-
C-2315	-	-	-
C-2316	-	-	-
C-2323	-	-	-
C-2053	4 dpc	-	10 dpc
C-2310	-	-	-
C-2330	-	-	-
C-2347	5 dpc	-	-
C-2152	3 and 5 dpc	-	8 and 10 dpc
C-2160	3, 4 and 5 dpc	-	8 and 10 dpc
C-2305	-	-	3 and 10 dpc
C-00888	5 dpc	-	8 and 10 dpc

pTOPO-P was done using *Nhe*I and *Eco*RI present on either sides of the insert in the vector backbone of pCR^{R}2.1-TOPO^R. The digestion resulted in the release of a 175 bp product only with pTOPO-R, confirming the presence of the genetic marker, i.e. *Nhe*I in the recombinant virus. Further sequencing analysis of pTOPO-R confirmed the presence of the Nhe1 site in r-O_1BFS, indicating that the virus isolated as a result of transfected RNA was indeed recombinant virus. Further, RT-PCR amplification of a 900 bp type O-specific product indicated that the progeny virus belonged to type O. The plaque morphology of r-O_1BFS and p-O_1BFS was similar.

Vaccine containing inactivated r-O_1BFS was prepared and its serological response and efficacy tested in guinea pigs and cattle. The immune response was comparable to the response against commercial vaccine containing O_1BFS. Further, the vaccine using r-O_1BFS could also be used as a booster vaccine as shown by the response in guinea pigs. Four vaccinated animals, each vaccinated with oil-adjuvant vaccine containing r-O_1BFS and p-O_1BFS, showed protective immune response at 21 dpv. On challenge with virulent FMDV, all four cattle in both the groups were protected, whereas the four unvaccinated cattle showed generalized clinical signs of FMD. The mean rectal temperatures of the control animals were higher compared with the vaccinated animals. Analysis of the clinical samples showed that virus replication was minimal in both the vaccinated groups as evident from the plasma virus concentration and the amount of virus excreted post challenge in the nasal secretions. No difference in the virus load was observed in the probang samples between the vaccinated groups.

FMDV isolates show marked genomic and antigenic variation, and the current vaccine strains were selected based on the serological spectrum with respect to a particular geographical area. Despite the successful application of inactivated vaccines in controlling the disease, research on FMD has been focused on the development of novel vaccines which could be manufactured without the fear of virus escape or the need for high-containment facilities

(Beck and Strohmaier 1987). Full-length cDNA clones from different FMDV strains allow the recovery of infectious virus particles, which are invaluable tools for the determination of virulence factors and for the elucidation of mechanisms involved in viral pathogenesis. Further, they give an opportunity to design new approaches for the development of recombinant FMDV marker vaccines (Zibert *et al* 1990; Fowler *et al* 2008). Despite the several advantages, not all the FMDV strains have been used for the construction of infectious cDNA clones (Liu *et al* 2004). The reasons could be the presence of a highly structured 5′ UTR and long poly [C] tract in the cardiovirus–aphthovirus group, which hampers the construction of full-length FMDV cDNA clones (Zibert *et al* 1990). After the first description of an FMDV type O infectious cDNA clone by Zibert *et al* (1990), several studies utilized this technology to investigate the biology of this virus (Zibert *et al* 1990; Reider *et al* 1993; Liu *et al* 2004). Many others have engineered recombinant viruses with altered antigenic determinants with downstream applications in vaccine development (Sáiz *et al* 2001; van Rensburg and Mason 2002: Fowler *et al* 2008), but no reports are available from India. This is the first report on generation of an infectious FMDV full-length cDNA clone and its use for vaccine development from India.

In conclusion, the availability of an infectious cDNA clone of FMDV may allow us to address many questions on the functions of viral gene products involved in infection and replication of this virus. It will also facilitate the design of a new approach to the development of chimeric FMDV viruses and marker vaccines.

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