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Megaprimer-mediated capsid swapping for the construction of custom-engineered chimeric foot-and-mouth disease virus

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Abstract Foot-and-mouth disease (FMD) is a highly contagious, economically important disease of trans-boundary importance. Regular vaccination with chemically inactivated FMD vaccine is the major means of controlling the disease in endemic countries like India. However, the selection of appropriate candidate vaccine strain and its adaptation in cell culture to yield high titer of virus is a cumbersome process. An attractive approach to circumvent this tedious process is to replace the capsid coding sequence of an infectious full-genome length cDNA clone of a good vaccine strain with those of appropriate field strain, to produce custom-made chimeric FMD virus (FMDV). Nevertheless, the construction of chimeric virus can be difficult if the necessary endonuclease restriction sites are unavailable or unsuitable for swapping of the capsid sequence. Here we described an efficient method based on megaprimer-mediated capsid swapping for the construction of chimeric FMDV cDNA clones. Using FMDV vaccine strain A IND 40/2000 infectious clone ($pA^{40/2000}$) as a donor plasmid, we exchanged the capsid sequence of $pA^{40/2000}$ with that of the viruses belonging to serotypes O ($n = 5$), A ($n = 2$), and Asia 1 ($n = 2$), and subsequently generated infectious FMDV from their respective chimeric cDNA clones. The chimeric viruses exhibited comparable infection kinetics, plaque phenotypes, antigenic profiles, and virion stability to the parental viruses. The results from this study suggest that megaprimer-based reverse genetics technology is useful for engineering chimeric vaccine strains for use in the control and prevention of FMD in endemic countries.

Keywords FMD virus · Megaprimer-PCR · Chimeric FMDV · Capsid Swapping

Introduction

Foot-and-mouth disease (FMD) remains one of the most economically important diseases of farm animals. The disease is highly contagious in nature and can affect a wide range of cloven-hoofed animals. The causative agent of the disease, FMD virus (FMDV) is a prototype member of the genus *Aphthovirus* within the family *Picornaviridae* [1]. The virus particle contains a single-stranded positive sense RNA genome of about 8.3 kb enclosed within an icosahedral protein shell consisting of 60 copies of each of the four capsid proteins VP4, VP2, VP3, and VP1. The inactivated conventional FMD vaccines have been used successfully for eradication of FMD in Europe, North America, and parts of South America. Furthermore, prophylactic vaccination with inactivated vaccine remains an important strategy for FMD control in endemic settings [2]. Since, FMDV evolve rapidly in different geographic areas, the efficacy of vaccination is affected by lack of cross-protection between serotypes and incomplete protection

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between some subtypes [3]. Therefore, there is a constant need to determine the effectiveness of in-use vaccine strain as well as selection of new custom-made candidate strains as per the epidemiological situation. However, screening of field isolates in order to select a candidate vaccine strain is generally cumbersome and laborious. Furthermore, adaptation of FMDV field isolates in cell culture to yield high titer of virus is an intricate and time-consuming process that may be associated with low success rate [4]. In addition, repeated passage of virus in cell culture may results in the change of antigenic structure.

To circumvent problem of possible antigenic variations of field virus during repeated passage in BHK-21 cell culture and speed-up the development of custom-made candidate vaccine stains, it is ideal to substitute the antigenic-coding region such as the capsid proteins of an infectious full-length cDNA clone of a vaccine strain with that of the desired field strain through reverse genetics technologies. The resulting chimeric virus retains the replication machinery of the backbone strain, while acquiring the antigenic determinants of field virus [5]. Furthermore, the chimeric viruses may also retain the pathogenic phenotype of the field strains [6]. In addition, as in case of FMDV, the capsid proteins determine the serotype specificity, using the full-genome length cDNA replicon of one serotype of FMDV, it is possible to develop recombinant virus of other serotype by replacing the structural protein coding genes [7]. The construction of chimeric infectious cDNA clone, however, can be difficult if the necessary endonuclease restriction sites are unavailable or unsuitable for capsid coding region swapping. At present, this requires sub-cloning and subsequent modification of internal cleavage sites before gene swapping. These time-consuming steps can be different in each case and require prior information of the nucleotide sequence.

A wide range of polymerase chain reaction (PCR)-based site-directed mutagenesis protocols have been established to introduce desired mutations (insertion, deletion, and substitution) into the target DNA sequence [8]. Among these methods, the megaprimer-PCR protocol is particularly useful because it is easy and relatively inexpensive [9, 10]. In the megaprimer method, in vitro DNA synthesis takes place in two successive steps: first, the target DNA fragment is amplified through PCR using two oligonucleotide primers and subsequently, the two strands of newly synthesized PCR amplicon are used as megaprimer to synthesize the whole plasmid in vitro to introduce the target DNA fragment. Each megaprimer anneals to the plasmid at the complementary site and then elongates from its 3′-end by a high-fidelity thermostable DNA polymerase [11, 12]. Subsequently, the original donor plasmid DNA is digested by restriction enzyme *Dpn*I and the *Dpn*I-resistant mutated DNA is rescued directly by transformation into chemically competent bacteria.

This manuscript demonstrates an alternative to the conventional restriction enzyme digestion and ligation for the construction of chimeric FMDV full-length cDNA clones using the megaprimer approach. We were able to construct and subsequently rescued infectious chimeric FMDV ($n = 9$), through the megaprimer-based capsid swapping protocol. The chimeric viruses exhibited similar infection kinetics, plaque phenotypes, antigenic profiles, and virion stability as compared to their parental viruses. The results from this study demonstrate that the megaprimer-based approach is suitable for rapid and reliable cloning of chimeric cDNA infectious clones without any prior information about the compatible restriction sites.

Materials and methods

Cells, viruses, and plasmid

FMDV-susceptible cell line BHK-21 was propagated in Glasgow minimum essential medium (GMEM) Sigma, USA) supplemented with 10 % fetal bovine serum (FBS). Virus stocks were prepared and titrated in BHK-21 cells using the plaque assay [13]. In addition, plaque assay was also performed on Chinese hamster ovary (CHO) cells (strain K1) propagated in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % FBS.

Viruses used in this study included five serotype O viruses {IND R2/1975 (vaccine strain), IND 120/2002, IND 32/2014, IND 70/2012, and IND 699/2013}; two serotype A viruses (IND 17/2009 and IND 437/2008) and two serotype Asia 1 viruses {IND 63/1972 (vaccine strain) and IND 148/2011}. Two out of the five serotype O viruses named IND 32/2014 and IND 699/2013 were isolated on IB-RS-2 cells and they have not been adapted to BHK-21 cells, while the rest of the viruses were adapted to BHK-21 cells. These viruses were obtained from the national FMDV repository maintained at ICAR-Project Directorate on Foot-and-mouth disease, Mukteswar, India. The GenBank accession numbers of the field viruses and vaccine strains capsid coding sequences are as follows: KP822947 (O IND R2/1975), KP822946 (O IND 120/2002), KP822942 (O IND 32/2014), KP822945 (O IND 70/2012), KP835578 (O IND 699/2013), HQ832592 (A IND 17/2009), HQ832591 (A IND 437/2008), KP822943 (Asia 1 IND 63/1972), and KP822944 (Asia 1 IND 148/2011).

Full-genome length infectious cDNA clone of the FMDV serotype A vaccine strain IND 40/2000 (pA$^{40/2000}$) [14] was used as donor plasmid for the construction of chimeric full-length cDNA clones through the megaprimer method.

Construction of chimeric full-length cDNA by megaprimer-mediated capsid swapping

Viral RNA was extracted using QIAmp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and reverse transcribed using Thermoscript[TM] Reverse Transcriptase enzyme (Invitrogen, USA) and FMDV-specific RT primer (NK61R). Megaprimer amplification was performed in two steps. In the first step, the region of the cDNA corresponding to the capsid coding region of the FMDV genome was amplified as a single fragment using a pair of universal primer L463F & NK61R [15] located in L and 2B genomic region of FMDV, respectively. Using the above primers combination, two microliters of each RT reaction was subjected to PCR (total reaction volume 50 µl) by an initial denaturation step (95 °C 2 min), followed by 30 cycles each consisting of 95 °C for 20 s, 55 °C 30 s, 70 °C 2 min, and final elongation (70 °C 10 min) using KOD Hot Start DNA polymerase kit (Novagen) according to manufacturer's instructions. Amplification product was electrophoresed on 1 % agarose gel and the amplicon was purified using Qiagen gel purification kit (Qiagen, Hilden, Germany).

In the second step, the purified PCR amplicon was used as megaprimer for in vitro amplification of the target plasmid. For this reaction, KOD Hot Start DNA polymerase Kit (Novagen) was used. The 50 µl amplification reactions contained 200 ng of megaprimer, 50 ng of donor plasmid (pA$^{40/2000}$), 5 µl of KOD Hot Start DNA polymerase buffer, 1.5 mM $MgSO_4$, 0.2 mM of each dNTPs, and 1 U of KOD Hot Start DNA polymerase enzyme. The thermal cycle program used was 95 °C for 2 min, 20 cycles of 95 °C, 20 s; 55 °C, 60 s; 70 °C, 5 min and final extension of 70 °C for 10 min. Following the PCR reaction, a digestion with 10 U of DpnI enzyme was performed at 37 °C for one hour in order to remove methylated donor plasmid. After enzymatic digestion, 10 ul of the reaction mixture was transformed into chemically competent XL-10 Gold[TM] (Agilent Technologies, USA) E. coli according to the manufacturer's instructions. Resulting clones were evaluated by multiplex PCR [16] and restriction enzyme digestion to verify that the selected clones contained the desired fragment corresponding to the capsid coding region of FMDV. Further, the positives clones were subjected to nucleotide sequencing in order to detect the presence of unintended mutation.

Transfection and rescue of virus from the chimeric full-length cDNA clones

Chimeric full-length cDNA clones were linearized at the NotI 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. The resultant RNA transcripts were treated with DNase I (Ambion, USA) to remove input plasmid. BHK-21 cells were transfected with these synthetic RNA by chemical transfection method as described previously (14). The transfected BHK-21 cells monolayer was washed and GMEM with 2 % FBS was added, and incubated up to 48 h with microscopic observation for cytopathic effect (CPE). The supernatants were used to infect fresh BHK-21 cell monolayer and incubated for up to 48 h at 37 °C. Viruses were subsequently harvested by repeated freeze-thawing and passaged 3–4 times on fresh BHK-21 cell monolayer using the procedure described before [17]. Rescued chimeric viruses were characterized using serotype-specific antigen-ELISA as described previously [18].

In order to confirm the antigenic similarity between the wild-type and recombinant chimeric FMDV, two-dimensional virus neutralization test (2D-VNT) was performed using FMDV serotype-specific bovine vacinal serum (BVS) as per the protocol described earlier [19]. The titre of the serotype-specific BVS against 100 $TCID_{50}$ of each virus was determined by regression analysis. The one-way serological relationship between the heterologous and homologous virus was expressed as 'r-value.' Serotype-specific BVS was obtained from the serum repository maintained at ICAR-Project Directorate on Foot-and-mouth disease, Mukteswar, India.

To determine the replication kinetics of rescued chimeric and parental viruses, one-step growth curve analysis was conducted. Four parental (O IND R2/1975, O IND 120/2002, A IND 17/2009, and Asia 1 IND 63/1972) and their respective chimeric viruses were arbitrarily selected along with the donor virus (A IND 40/2000) to study the replication kinetics. BHK-21 cell monolayers were infected with the selected parental and chimeric FMDV at a multiplicity of infection (m.o.i) of 2, washed extensively at 1 h post-infection with PBS (pH 6), and then incubated at 37 °C for 2, 6, 10, 14, and 18 h. The titres at various time points post-infection were determined by the plaque assay [13].

Stability of chimeric FMDV to different biophysical conditions

FMDV serotype O, Asia 1 vaccine strains (O IND R2/1975 and Asia 1 IND 63/1972, respectively), and serotype A field strain (A IND 17/2009) and their respective derived chimeric viruses (vO$^{R2/1975}$-A, vAsia1$^{63/1972}$-A and vA$^{17/2009}$-A, respectively) were selected arbitrarily to study their stabilities to different biophysical conditions such as temperature, pH, and salt concentrations. Viruses were concentrated using PEG-8000 (Sigma-Aldrich) and purified by cesium chloride centrifugation method [20]. Following

fractionations, peak fractions corresponding to 146S FMDV particles were pooled and titrated on BHK-21 cell monolayer. After diluting the purified virion particles in TNE buffer [100 mM Tris (pH 7.4), 10 mM EDTA,150 mM NaCl], the stability of FMDV at different temperatures (25–55 °C), pH values (9.0–6.0), and NaCl concentrations (50–1600 mM) was determined using a procedure described before (5).

Results

Megaprimer mediated full-genome length plasmid amplification

A megaprimer approach was applied to replace the capsid coding region of the full-length cDNA clone of FMDV A IND 40/2000 (pA$^{40/2000}$) with that of the respective capsid coding sequence of the desired FMDV. A schematic diagram of the megaprimer amplification is shown in Fig. 1. A standard PCR was performed using a pair of common primer for amplification of the capsid coding region (∼2.2 kb) of the FMDV serotypes O, A, and Asia 1. Subsequently, the PCR amplicon served as megaprimer in the second amplification reaction during which each strand of PCR amplicon was denatured, annealed to the original donor plasmid (pA$^{40/2000}$), and extended with the high-fidelity DNA polymerase in order to generate chimeric full-

length cDNA clone containing the capsid coding sequence of the virus of interest in the backbone of the donor plasmid. After the DpnI digestion of the donor plasmid, an aliquot of amplified product was transformed into chemically competent XL10-Gold E. coli cells. The number of bacteria colonies after transformation varied from 8–35 per plate (Table 1). Insertion of the respective capsid coding sequence was confirmed by mPCR, restriction enzyme digestion, and sequencing (data not shown). From the analyzed clones, 73–100 % carried the insert of interest (Table 1). Using the megaprimer-based cloning method, we generated chimeric full-length cDNA plasmids for the 9 viruses and these are named as pO$^{R2/1975}$-A, pO$^{120/2002}$-A, pO$^{32/2014}$-A, pO$^{70/2012}$-A, pO$^{699/2013}$-A, pA$^{17/2009}$-A, pA$^{437/2008}$-A, pAsia1$^{63/1972}$-A, and pAsia1$^{148/2011}$-A.

Rescue and characterisation of FMD viruses from chimeric cDNA plasmid

Through the megaprimer method, we constructed chimeric cDNA clones containing the entire capsid coding region of a different FMDV (either serotype O, A, or Asia 1) in the defined genetic backbone of an infectious pA$^{40/2000}$ cDNA clone. The in vitro synthesized RNA transcripts from the chimeric cDNA plasmids were transfected into BHK-21 cell monolayer and viable chimeric viruses were recovered. The recombinant viruses derived from the infectious chimeric cDNA clones were designated as vO$^{R2/1975}$-A, vO$^{120/}$

Fig. 1 A schematic diagram of the megaprimer-mediated capsid swapping for the construction of chimeric full-length cDNA plasmid. Megaprimer amplification was performed in two steps. First, the capsid coding region of field viruses was PCR amplified and gel-purified (Step I). In the second step, the PCR amplicon was used as megaprimer for the in vitro amplification of the donor plasmid (pA$^{40/2000}$) (Step II). Subsequently, DpnI digestion removes the donor plasmid, and the in vitro synthesized chimeric cDNA was used to transform chemically competent E. coli
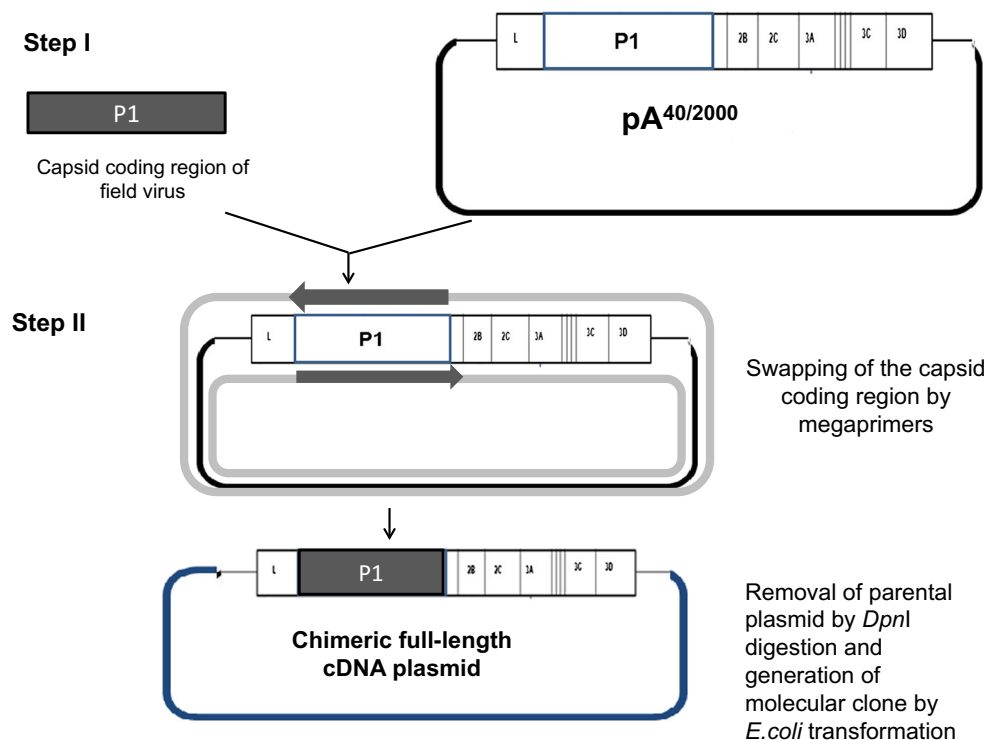
Table 1 Cloning efficiency of megaprimer-based method: numbers of colonies and of clones with correct insert

| FMDV capsid coding sequence	No. of bacteria colonies	Correct clones/number analyzed (% of positive clones)
O IND R2/1975	15	10/12 (83.3 %)
O IND 120/2002	22	11/12 (91.6 %)
O IND 32/2014	10	8/10 (80 %)
O IND 70/2012	28	12/15 (80 %)
O IND 699/2013	8	8/8 (100 %)
A IND 17/2009	28	14/15 (93.3)
A IND 437/2008	25	11/15 (73.3 %)
Asia 1 IND 63/1972	35	14/15 (93.3 %)
Asia 1 IND 148/2011	24	12/12 (100 %)

^{2002}-A, vO$^{32/2014}$-A, vO$^{70/2012}$-A, vO$^{699/2013}$-A, vA$^{17/2009}$-A, vA$^{437/2008}$-A, vAsia1$^{63/1972}$-A, and vAsia1$^{148/2011}$-A.

Plaque morphologies, growth kinetics, and antigenic properties of the recombinant rescued viruses were examined to determine whether they resembled those of the parental viruses. As illustrated in Fig. 2, serotype O, A, and Asia 1 viruses, and their respective chimeric viruses produced large plaques (6–8 mm) and medium (3–5 mm) plaques on BHK-21 cells. In CHO-K1 (glycosaminoglycan positive) cells, both the parental and derived chimeric viruses produced small (<2 mm) and medium (3–5 mm) plaques at 40 h post-infection. Although the chimeric viruses (vO$^{32/2014}$-A and vO$^{699/2013}$-A) containing the capsid coding region of field isolates (O IND 32/2014 and O IND 699/2013, respectively) yielded medium plaques on BHK-21 cells, they were unable to produce plaques on CHO-K1 cells similar to the parental field strains (Fig. 2). Therefore, the comparable ability to infect the cultured cells suggested that capsid coding region of chimeric viruses retained the characteristics of their respective parental viruses.

The one-step growth curve study of both parental and genetically engineered chimeric viruses illustrates that the growth kinetics of the chimeric viruses (vO$^{R2/1975}$-A, vO$^{120/2002}$-A, vA$^{17/2009}$-A, and vAsia1$^{63/1972}$-A) were similar to that of the recombinant serotype A IND/40/00 virus (Fig. 3a) and their parental viruses (Fig. 3b). Following infection of BHK-21 cells at an m.o.i of 2, all the viruses (both recombinant and parental) yielded high and comparable titer at 12–16 h post-infection (Fig. 3).

Fig. 2 Plaque morphologies of the parental and chimeric FMDV obtained using the monolayers of BHK-21 and CHO-K1 cells. Cells were infected with the indicated viruses and were incubated with agar overlay for 34–40 h prior to staining with 0.1 % crystal violet. The parental and their respective chimeric viruses produced large- (6–8 mm) and medium (3–5 mm)-sized plaques on BHK-21 cells, while on CHO-K1 cells the parental and chimeric FMDV produced medium- and small-sized (<2 mm) plaques. Due to the limitation of space, plaque morphologies of six out of the nine chimeric rescued FMDV have been illustrated in this figure
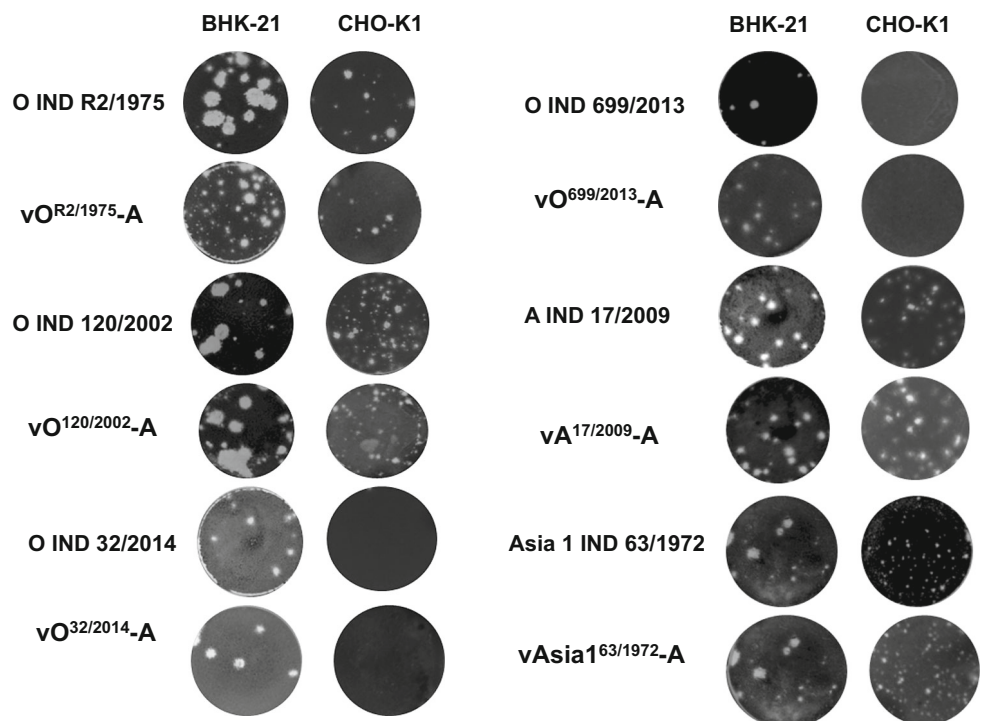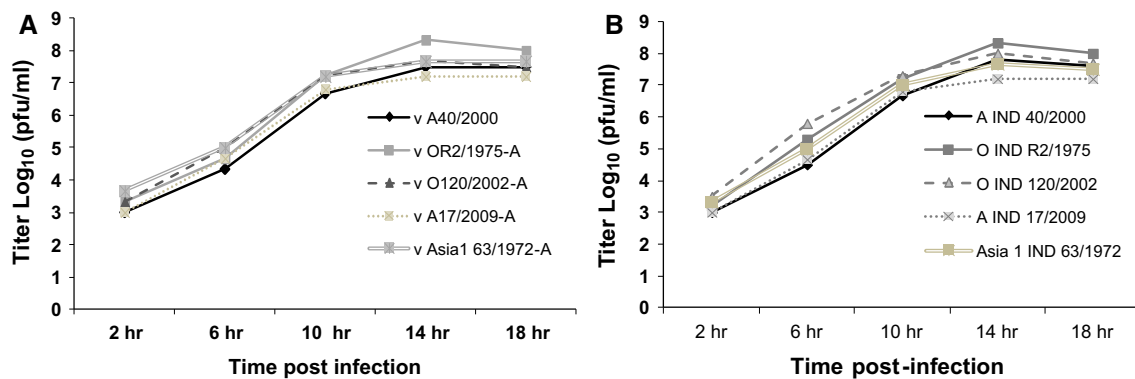
Fig. 3 One-step growth kinetics studies in BHK-21 cells. The average log virus titers are shown at different hours post-infection for the recombinant viruses named vA 40/2000, vO$^{R2/1975}$-A, vO$^{120/2002}$-A, vA$^{17/2009}$-A, and vAsia1$^{63/1972}$-A (**a**), and the respective parental viruses named A IND 40/2000, O IND R2/1975, O IND 120/2002, A IND 17/2009, and Asia 1 IND 63/1972 (**b**). The average virus titers obtained from the triplet wells at different time points post-infection (2, 6, 10, 14, and 18 h) are indicated on the graph

Further, we compared the antigenic profile of the parental and the genetically engineered chimeric viruses using the BVS raised against the vaccine strains of the respective serotypes (O IND R2/1975, A IND 40/2000, and Asia IND 63/1972). Based on the 2D-VNT assay results, the r-values indicated that the sera reacted similarly to both the parental and chimeric viruses (Table 2), suggesting the absence of variation in the antigenic determinants on the chimeric virions. Therefore, the results from the plaque phenotypes, growth kinetics, 2D-VNT assay demonstrate that antigenic

characteristics and receptors preferences of the parental viruses were transferred to the recombinant chimeric virus through the megaprimer-based cloning method.

Stability of chimeric and parental FMDV to different biophysical conditions

Biophysical stability of FMDV has been correlated with the protective nature of FMD vaccines [21]. Therefore, the biophysical stability of serotypes O, A, and Asia 1 chimeric

Table 2 Comparison of the antigenicity of FMDV serotypes O, A, and Asia 1 parental and their respective chimeric viruses as measured against the serotype-specific bovine vaccinal serum

Viruses	r values		
	Bovine vaccinal serum (BVS)		
	Anti-O R2/1975	Anti-A 40/2000	Anti-Asia 1 63/1972
O R2/1975	1	n.d	n.d
vO$^{R2/1975}$-A	0.96	n.d	n.d
O 120/2002	0.68	n.d	n.d
vO$^{120/2002}$-A	0.76	n.d	n.d
O 32/2014	0.8	n.d	n.d
vO$^{32/2014}$-A	0.72	n.d	n.d
O 70/2012	0.86	n.d	n.d
vO$^{70/2012}$-A	0.94	n.d	n.d
O 699/2013	0.36	n.d	n.d
vO$^{699/2013}$-A	0.3	n.d	n.d
A 17/2009	n.d	0.32	n.d
vA$^{17/2009}$-A	n.d	0.35	n.d
A 437/2008	n.d	0.41	n.d
vA$^{437/2008}$-A	n.d	0.38	n.d
Asia 1 63/1972	n.d	n.d	1
vAsia1$^{63/1972}$-A	n.d	n.d	1
Asia 1 148/2011	n.d	n.d	0.64
vAsia1$^{148/2011}$-A	n.d	n.d	0.72

n.d not done

viruses (vO$^{R2/1975}$-A, vA$^{17/2009}$-A, and vAsia1$^{63/1972}$-A) and their respective parental viruses (O IND R2/1975, A IND 17/2009, and Asia 1 IND 63/1972) to different temperature, pH conditions, and ionic strength was evaluated. When incubated at temperatures ranging from 25 to 55 °C, a gradient decrease in virus infectivity titre was detected for chimeric viruses, similar to those of the parental viruses (Fig. 4a). No infectious particles were detected either for chimeric or parental viruses after incubation at 55 °C for 30 min (Fig. 4a). Furthermore, after extended incubation at 4 °C infectious titer was detected for both the chimeric and parental viruses up to 28 days (Fig. 4b).

The stability of the chimeric and parental viruses was evaluated at pH values ranging from 6.0–9.0. Both the chimeric and parental viruses displayed a comparable decrease in viral titer when the pH of the buffered solutions was reduced from 9.0–6.0 (Fig. 5a). Further, a similar capsid stability of chimeric and parental viruses was observed at various NaCl concentrations ranging from 50 mM to 1.6 M (Fig. 5b). Therefore, the chimeric and parental viruses did not show any significant differences with respect to their biophysical properties.

Discussion

Basic research and vaccine development have been expedited by the generation of recombinant FMDV from the full-genome length cDNA plasmid through reverse genetics technologies. However, the established full-length infectious cDNA clone systems for FMDV require prior cleavage of amplicons with terminal restriction enzymes prior to insertion into appropriately cleaved plasmid vector [17, 22–25]. To make this procedure straightforward, the current study was undertaken to develop an efficient and

reliable capsid coding region swapping method that would allow for the quick construction of FMDV full-length cDNA clones encoding chimeric viral genomes. Instead of the use of restriction enzymes and ligases, a two-step mutagenesis procedure was developed in this study for the replacement of capsid coding region in the donor plasmid (pA$^{40/2000}$) with that of the capsid sequence of the FMDV of interest. Although, earlier studies showed that larger megaprimers were exchanged less efficiently than shorter megaprimers [26, 27], the results from this study have demonstrated an efficient exchange of large fragments (∼2.2 kb) encoding the structural proteins of FMDV.

The primer binding sites of the PCR primers, L463F (5′-ACCTCCRACGGGTGGTACGC-3′) and NK61R (5′-GACATGTCCTCCTGCATCTG-3′), used for the amplification of the capsid coding region of FMDV were found conserved across all the three serotypes (O, A, and Asia 1) circulating in India (Figure S1). Therefore, the current megaprimer method could be useful for the universal cloning of chimeric cDNA plasmid for FMDV serotypes O, A, and Asia 1. Furthermore, the above primer binding sites were also found conserved for FMDV serotype C (data not shown), therefore, theoretically the megaprimer-based capsid swapping procedure could be used for the construction of chimeric FMDV serotype C. However, the current in-use primers (L463F & NK61R) may not be used for the construction of chimeric SAT viruses, as the primer binding sites are not found conserved across the SAT serotypes of FMDV.

A potential constraint with all PCR-based cloning methods is their susceptibility to unwanted mutation during in vitro amplification of PCR product. However, as compared to the standard PCR, the predicated mutation rate for the megaprimer-based PCR could be extremely low for several reasons. First, the newly synthesized strand of each
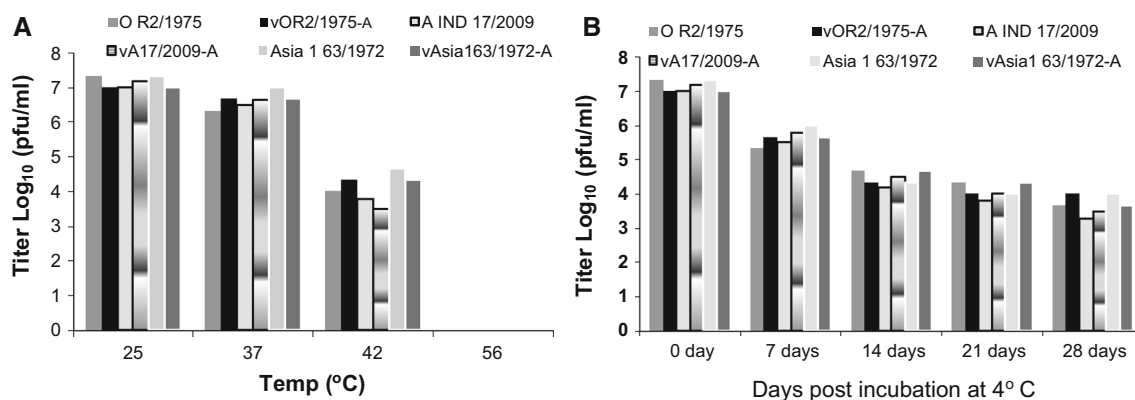

Fig. 4 Thermal stability of the purified 146S virion particles of the O IND R2/1975, A IND 17/2009, Asia 1 IND 63/1972 viruses and their respective chimeric viruses (vO$^{R2/1975}$-A, vA$^{17/2009}$-A, and vAsia1$^{63/1972}$-A, respectively) at different temperatures. Purified 146S virion

particles were incubated at the indicated temperature for 30 min (**a**) and at 4 °C for 28 days (**b**). All the samples were titrated by plaque assay on BHK-21 cell monolayers and average log titers of two experiments are plotted
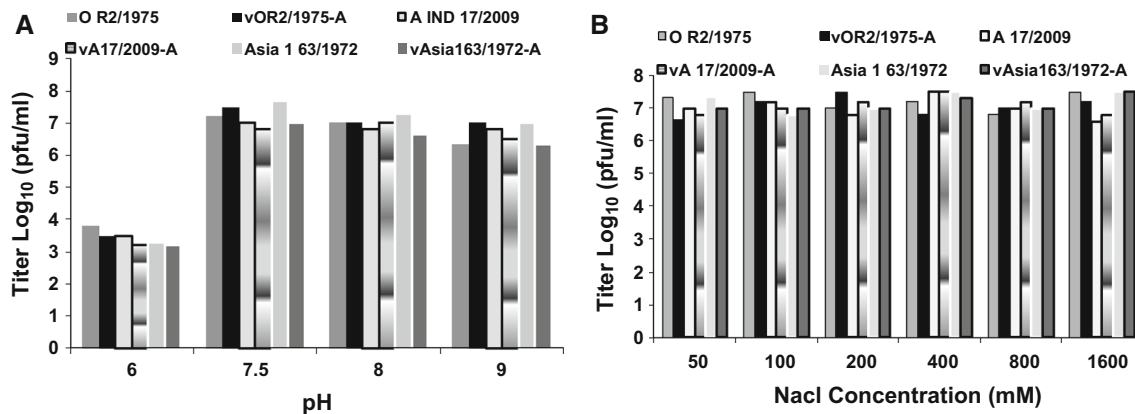
Fig. 5 pH (**a**) and ionic stability (**b**) the purified 146S virion particles of the O IND R2/1975, A IND 17/2009, Asia 1 IND 63/1972 viruses and their respective chimeric viruses (vO$^{R2/1975}$-A, vA$^{17/2009}$-A, and vAsia1$^{63/1972}$-A, respectively). All the samples were titrated by plaque assay on BHK-21 cell monolayers and average log titers of two experiments are plotted

round of PCR cannot serve as template for the following round of amplification because it contains nick at the initiation site of each megaprimer and therefore, in the megaprimer-PCR, there are two cyclic linear primer extensions from the plasmid template strands, but not an exponential amplification as in standard PCR [28]. Second, the megaprimer-based PCR requires fewer reaction cycles (20 cycles) than the standard PCR, which reduces the potential mutation rate. In addition, the use of high-fidelity DNA polymerase enzyme further reduces the chances of unwanted mutation in the megaprimer-PCR protocol.

The chimeric viruses containing the capsid coding sequence of different viruses in the serotype A background were rescued from their respective chimeric cDNA clone through reverse genetics technology. Furthermore, we have demonstrated that the chimeric FMDV retained the plaque morphology, infective kinetics, and antigenic profiles of their respective parental isolates from which they were derived. With respect to the usage of cellular receptors, the chimeric serotype O field viruses (vO$^{32/2014}$-A and vO$^{699/2013}$-A), grew in BHK-21 cells following amplification in cell culture; however, they were unable to produce plaques in integrin-deficient CHO-K1 cells, similar to that of their respective parental viruses. This corroborates the earlier reports that the field viruses may not utilize GAG receptors for cell entry [29, 30].

FMD virion stability is of utmost importance during the vaccine manufacturing process as the maintenance of intact 146S particles is directly related to the vaccine efficacy [4, 21]. During the present study, through limited biophysical analysis, it was observed that both the parental and chimeric viruses showed a comparative stability at a range of temperature, pH, and ionic strength. Therefore, the swapping of capsid coding sequence of one virus on the genetic background of another FMDV through megaprimer method did not alter the biological properties of the chimeric virus.

In conclusion, we established a simple and easy method which can be used for the generation of custom-engineered chimeric FMDV, independent of restriction cleavage and ligation reaction. In future, megaprimer-based domain swapping method could be useful to study the molecular pathogenesis of FMDV.

Compliance with ethical standards

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

Animal ethics This study complied with international standards for animal welfare.

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