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Creation of leaderless FMDV replicon for development of replication defective virus (leaderless FMDV): A strategy towards the development of attenuated vaccine with marker facility

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Leader protease (L^{pro}) of foot-and-mouth disease virus (FMDV) which is essential for viral replication and pathogenicity in host is found in two forms, Lab and Lb with similar activity but, both differing only at amino-termini with separate initiation codons, 84 nucleotides apart. After translation of the genomic RNA into single polyprotein, the L protease is released autocatalytic from the N terminus and cleaves the p220 subunit of the eukaryotic initiation factor 4F complex resulting in the shut off host protein synthesis, an essential step for viral pathogenicity. We exploited this function for development of attenuated virus by deleting the gene encoding Lb protease from FMDV Asia 1(63/72) cDNA replicon (Joshi *et al,* 2013) by PCR mutagenesis approach. The deletions and intactness of the frames were confirmed by sequence analysis. P1-2A polyprotein gene of FMDV 'O' was inserted into the replicon and the full length construct was studied for virulence to baby hamster kidney (BHK) 21 cells. Vaccinia expressing T7 polymerase was used for *in vitro* RNA generation and infection. The lower cytopathic effect (cpe) as observed by reduced replication efficiency confirmed the effect of Lb deletion when compared with the construct with no deletion.

Keywords: Cloning, expression, Lb-protease deletion, T7 polymerase, FMDV virulence, BHK cells, attenuation

Introduction

The leader proteinase (L^{pro}) of foot-and-mouth disease virus (FMDV) is a papain-like proteinase[1] that plays an important role in FMDV pathogenesis. The inhibitor and site-directed mutagenesis studies have confirmed that it belongs to this class of proteinases[2-3]. L^{pro} is involved in the inhibition of the type I interferon (IFN) response during virus infection and Lb^{pro}, a shorter form of L^{pro}, has been reported to have deubiquitinating activity[4].

The L gene is present at the 5' end of the open reading frame (ORF) and contains two in-frame initiation codons which are 84 nucleotides apart and encodes proteins called as Lab and Lb[5]. All FMDV serotypes carry these two initiation codons and are detected in *in vitro* translation and in infected cells[6-7] and the first AUG (at the start of Lab) is not always required to initiate replication efficiently[8]. The L protein auto catalytically cleaves itself from the N-terminus of the elongating polyprotein in trans[9], exposing the N-terminus of the capsid precursor protein P1, permitting its N-terminal myristoylation[10]. L protease leaves the p220 subunit of eukaryotic initiation factor 4F (eIF-4F)[11-12] that results in the shut off of cap-dependent host mRNA translation[13].

The classical viral vaccines using inactivated viruses have the limitations of poor and short duration of immune response and hence not successful in some cases[14], such as human immune deficiency virus or herpes simplex virus. Live attenuated virus vaccines can overcome these limitations, however naturally attenuated viruses have the well-known problem of instability[15] as the mutation at a limited number of sites could easily revert to virulence. Therefore an alternative approach to attenuate the virus is by genetic modified by creating necessary deletions in the genome. Attempts to attenuate FMDV by deletion of the poly (C) tract near the 5' end of the genome[17] have been made with no success. Attenuated cardio virus has been produced using classical genetics engineering techniques. A genetically modified variant of FMDV lacking the coding region for the

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leader (L) proteinase was developed[18]. The leaderless virus was shown to be attenuated both in tissue culture and in suckling mice. However, in order to develop polyvalent vaccine, this approach needs to be repeated with every serotype.

Here we report the generation of leaderless FMDV replicon as a first step and study its application in generating live attenuated FMD vaccines with desired serotype.

Materials and Methods

Deletion of Lb-Protease Gene from the Replicon

Full length cDNA clone of FMDV Asia 1 63/72 (vaccine strain) is available in the laboratory (Suryanarayana *et al*, 2003). A replicon of the same was developed by creating restriction sites and deleting the capsid coding region, P1-2A and was used for Lb protease gene deletion. The sequence corresponding to part of 5'UTR and part of 2B carrying unique restriction sites (*Xba*I and *Bgl*II) was amplified as two fragments which are, 1. Part of UTR with unique *Xba*I site to beginning of VP4 and 2. Beginning of VP4 to part of 2B with unique *BglII* site. The primers used for this purpose are shown below:

1 UTR (*Xba*I) (L): CCA GGT CTA GAG GGG TGA CAC CTT 24 mer
2 UTR-VP4 link(R) : TTG CCG GGC TGG ATT GCC CGG CTC CCA TTT TCT CTT GTG TCC GTA GAA GA 50 mer
3 VP4-2B (L): TCT TCT ACG GAC ACA AGA GAA AAT GGG AGC CGG GCA ATC CAG CCC GGC AA 50 mer
4 2BR (R) *Hind*III/*Bgl*II: GCA AGC TTG CGA GTC GGA GAT CTT CTT CAC 30 mer

Both the fragments were annealed at the complementary region and treated with Klenow for primer extension in the presence of dNTPs. The primer extended DNA was used as template to amplify UTR-2B fragment with deleted Lb-protease gene.

Infectivity Studies on the Transcripts of Full-Length Constructs

Insertion of Green Fluorescent Protein (GFP) Gene into Replicon for *In Vitro* Studies

In order to confirm the defective replication thereby attenuation *in vitro* we have inserted GFP gene in frame in the place of structural protein genes (P1-2A). Level of fluorescence in the transfected BHK cells indicates the level of attenuation.

GFP Cloning in L-Deleted Asia 1 63/72 cDNA

The 2A region of FMDV plays a pivotal role in viral replication, therefore, it is essential to include 2A gene along with the green fluorescent protein (GFP) gene. To do this we first cloned P1-2A and a part of 2B (2.6 kb) region of Asia 1 63/72 into pBKS+ plasmid at *Eco*RI and *Xho*I and this was further used for cloning GFP sequence.

The GFP sequence was amplified from a plasmid carrying GFP gene (pCGFP) using the primers

GFP(L)/ *Eco*RI- *Bam*HI: GCGGAATTCCATGGA TCCAGTGAGCAAGGGCGAGGAGCT

GFP(R)/*Nhe*I: GCGGAATTCTTACTTGTACAG CTCG TCCAT

The amplified product (750 bp) was purified from agarose gel and inserted into pBKS+ vector carrying P1-2A gene of Asia 1 63/72 at *Eco*RI and *Nhe*I site thereby replacing P1 region with GFP while retaining 2A. Finally, the GFP along with 2A region was cloned into FMDV genome lacking L region at *Bam*H1 site. The full-length cDNA with GFP gene was transcribed *in vitro* using transcription kit. The RNA was purified and about 10 μg of each RNA transcript was transfected into BHK cells as per standard protocol using transfectamine 2000 reagent (Invitrogen, USA)

Generation of Gene Deleted FMDV Chimeric 'O' cDNA Construct

Asia 1 replicon with L-deletion was used to generate FMDV type 'O (chimeric 'O'). The methodology to generate full length cDNA construct with P1-2A of FMDV 'O' R2/75 (Indian vaccine virus) was described elsewhere (Joshi *et al*, 2013). Using this approach P1-2A of 'O' was introduced into the Lb gene deleted replicons at created *Bam*HI site and the un-deleted replicon with P1-2A 'O' served as a positive control.

Development of Biosynthetic Viruses with Gene Deleted Constructs

BHK 21 clone 13 cell line was grown in DMEM with 10% fetal bovine serum (FBS, HI Media) at 5% CO_2 tension in 6 well tissue culture dishes to 90% confluence. The cells were washed thrice with DMEM without serum and the cells were infected with recombinant vaccinia virus vTF7-3 (Fuerst *et al*, 1986) expressing T7 DNA dependent RNA polymerase at multiplicity of infection (moi) 10 at 37°C for 45 min. The cells were washed once, with Opti-MEM (Invitrogen, USA) and transfected with the plasmid, linearized at the restriction site *Not*1, downstream of

poly (A) tract, using lipofectamine-2000 as per manufacturer's protocol. Briefly, 2 to 4 µg of linearised DNA in 250 µl of Opti-MEM was mixed with 6 µl of lipofectamine in 250 µl Opti-MEM, incubated for 20 min at room temperature and the DNA- liposome mixture was layered slowly on the mono layer. The cells were incubated at 37°C with 5% CO_2 tension for 4 hr. Later the transfection mixture was replaced with DMEM containing 0.5% FBS and further incubated for 48 hrs. The infected cells (with vaccinia and FMDV) were harvested by two freeze-thaw cycles, treated with 1% (v/v) chloroformate 4°C and the supernatant (500 µl) after centrifugation at 10,000 g for 10 min was used for subsequent passage in to BHK-21 cells in 6-well cell culture dish. After the first passage (after 24 hrs) the total RNA was extracted from infected cells with trizol (Invitrogen) reagent as per the manufacturer's protocol and 1 µg of the total RNA was used for subsequent transfection in to BHK-21 cells in 6-well cell culture dish. This step helps inner moving the vaccinia virus in the infected culture. The cells were observed for cytopathic effect (cpe) for 24 hr post infection (PI). The FMDV in the cell supernatant was collected, 500 µl of the supernatant was passaged in BHK cells in 25 cm 2 cell culture flasks. The virus from each passage was collected and stored at -70°C after chloroform treatment as described before.

Characterization of the Biosynthetic Viruses

The virus was studied for the absence of the deleted genes and replication efficiency by RT/PCR using specific primers, immunoblot analysis of the virus infected cell culture supernatants and by infectivity studies in cell culture.

Results

Generation of Lb deleted FMDV Asia 1 Replicon

The replicon minus Lb gene was transferred into *E. coli* Top 10 cells. A number of transformants were seen on ampicillin plate and colonies were screened by colony PCR using IRES (L) and VP4-UTR link (R) primers. Since, the VP4-UTR link primer cannot bind undeleted gene the amplification is expected only with the deleted full length replicon, but not with un-deleted one. As seen in the Figure 1, amplification of 850 bp fragment (lane 2) in the case deleted construct and around 1.3 kb in the case of undeleted construct (lane 1) confirms the deletion of the Lb fragment of around 500 bp between IRES to VP4.

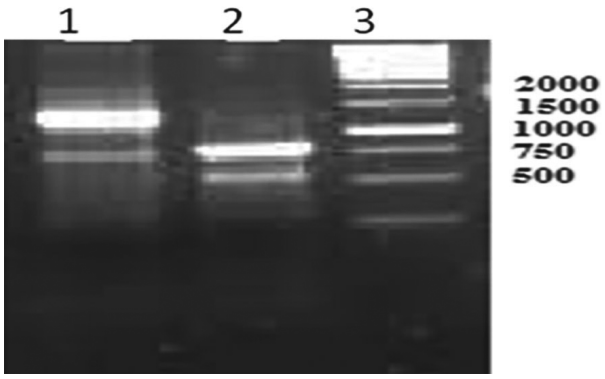

Fig. 1 — Analysis of the PCR amplified products from Lb un-deleted and deleted FMDV replicons, Lane 1 & 2 — amplified products from undeleted and deleted constructs respectively. Lane 3 — 1.0 Kb ladder

The Lb-deleted replicon pFLA(v)-L (Fig. 2) was used for further studies to develop an attenuated virus.

The sequence was subjected for BLAST search (Fig. 3) using NCBI programme for further confirmation of the deleted regions.

Insertion of GFP Gene in PFLA (v)-L

GFP gene was inserted in to pFLA (v)-L and studied for the insertion as described under materials and methods. The colonies were initially screened by colony PCR. Of the three colonies that were positive by colony PCR, plasmids from two were digested with *Bam*HI and the fragments analyzed by agarose gel electrophoresis. As seen in the Figure 4 there is a release of around 1.0 kb fragment after *Bam*HI digestion of the plasmids from both the L deleted (lane 1, 2) clones. The size of the GFP gene is 716 bp, along with the downstream 2A and part of 2B the total size of the insert would be 1.0 kb which is the observed size. GFP insert in both the cases is of the same size indicating both the constructs are intact as far as GFP-2A-2B insert is concerned. The plasmids from the confirmed clones were purified and used for replication studies in BHK cells.

Replication Efficiency of GFP Gene Inserted Replicons with Lb Deletions

RNA transcripts of *Not*I linearized constructs were synthesized *in vitro* using Thermo Fischer's high yield RNA transcription kit. After DNAse treatment to remove template the RNA transcript in each case was purified, re-suspended in DEPC treated water and studied for biological activity in BHK 21 cells. The expression of GFP was in transfected cells was monitored 48 hr post transfection (Fig. 5). Though the intensity is less the number of cells showing fluorescence were higher in the case of undeleted

TCTAGAGGGGTGACACCTTGTACTGTGTTTGACTCCACGCTCGGTCCACTAGCGAGTGTT
AGTAGTAGTACTGTTGCTTCGTAGCGGAGCATGACGGCCGTGGGAATCCCTCCTTGGCAA
CAAGGACCCACGGGGCCGAAAGCCACGTCCTGGAGGACCCGTCATGTGTGCAACCCCAG
CACGGCAGCTTTATTATGAAACCCACTTTAAGGTGACACTGATACTGGTACTCAACACTG
GTGACAGGCTAAGGATGCCCTTCAGGTACCCCGAGGTAACACGCGACACTCGGGATCTG
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GGCGACCGGAGGCCGGCGCCTTTCCTTTGACCACTACTGTTTACATGAACATGACCGACT
GCTTTATCGCTTTGTTGTACGCCATCAGGGAGATCAAAGCACGACTTCTTCTACGGACAC
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CAACACTGGAAGCATCATTAACAACTACTACATGCAGΔCAATACCAGAATTCCATGGATC
CGACTTTAACCGGTTGGTTACCGCGTTTGAGGAATTGGCCACTGGGGTGAAGGCTATCAG
GACCGGTCTCGACGAGGCCAAGCCCTGGTACAAGCTCATCAAACTCCTAAGCCGCCTGTC
ACGTATGGCCGCTGTAGCAGCACGGTCCAAGGACCCACTCCTTGTGGCCGTCATGCTGGC
TGATACCGGCC

Fig. 2 — Ladder sequences corresponding to IRES to 2B region of the pFLA(v)–L replicon. The nucleotide sequence of 485 bp corresponding to IRES is with red colour, The nucleotide sequence corresponding to part of undeleted leader (La) of 87 nts is shown with green, The black coloured sequence corresponds to partial 2B gene. Lb deletion is shown with symbol Δ. Created *Bam*HI site for P1-2A insertion and *Xba*I at the beginning of IRES in the replicon are underlined.

replicon (Fig. 5B) than L replicon (Fig. 5D). As seen in the Figure 5, when the transfected cells were visualized under visible light (Fig. 5A and C) there was a visible difference in the cell morphology. Cells transfected with undeleted replication (Fig. 5C) showed more rounding and bulging appearance as compared to pathogenicity in the case of deleted construct (Fig. 5A) indicating the reduced replication efficiency in the case of deleted construct.

Infectivity Studies on Full Length Chimeric 'O' cDNA with Lb deletion or without

Insertion of FMDV Serotype 'O' (R2/75, Vaccine Strain) P1-2A into Gene Deleted Replicons

Polyprotein coding region P1-2A of FMDV 'O' was released from pBO-P1-2A with *Bam*HI and cloned into L^{pro} gene- deleted full length Asia 1 63/72 replicon as described under methods. Plasmids from transformants were purified and screened for the presence of inserted P1-2A by *Bam*HI digestion and agarose gel electrophoresis. Since the P1-2A was cloned in a created single *Bam*HI site, initially the transformants were screened for the presence of insert in positive orientation by PCR using reverse primer corresponding to 3A and forward primer corresponding to VP 4. The PCR positive colonies (data not shown) were further studied for the presence of 'O' P1-2A insert by restriction enzyme digestion. As shown in the Figure 6, of the four colonies (lane 1, 2, 3, 5) screened for the release of 2.8 kb fragment one showed the release of the insert of size 2.8 kb upon digestion with *Bam*H1 (Fig. 6, lane 5). The positive L-deleted full length 'O' construct was designated as pBFLOΔLb.

Infectivity Studies on the Transcripts of Full Length Constructs

In order to achieve *in vivo* transcription and subsequent infection in BHK-21 cells, we have studied the infectivity of the plasmid DNA after transfection in genetically modified recombinant BHK cells expressing T7 RNA polymerase. The efficacy of the virus rescue in this case was compared with the cells co-infected with the full length DNA construct along with the recombinant vaccinia virus expressing T7 RNA polymerase. In the case of vaccinia system the recombinant vaccinia virus was allowed to infect BHK cells prior to transfection with the *Not* I linearized DNAs as described under materials and methods. The efficiency of virus rescue in the case of cells (clone 2/1) expressing T7 RNA polymerase was studied by transfecting with *Not*I linearized DNAs (Fig. 7). More than 80% cpe was observed in the case of FLO(-Lb) at First passage (B, C). However, the efficiency of virus rescue is high with vaccinia system (B) as compared to T7 RNA polymerase expressing cell system (C). This may be due to the higher level of RNA polymerase production in the case of recombinant vaccinia virus system.

Range1 736-1221GenBankGraphics match First match

793 bits(429) 0.0 439/444(99%) 0/444(0%) Plus/Plus
Query 1 TCTAGAGGGGTGACACCTTGTACTGTGTTTGACTCCACGCTCGGTCCACTAGCGAGTGTT
Sbjct 736 TCTAGAGGGGTGACACCTTGTACTGTGTTTGACTCCACGCTCGGTCCACTAGCGAGTGTT
Query 61 AGTAGTAGTACTGTTGCTTCGTAGCGGAGCATGACGGCCGTGGGAATCCCTCCTTGGCAA
Sbjct 796 AGTAGTAGTACTGTTGCTTCGTAGCGGAGCATGACGGCCGTGGGAATCCCTCCTTGGTAA
Query 121 CAAGGACCCACGGGGCCGAAAGCCACGTCCTGGAGGACCCGTCATGTGTGCAACCCCAGC
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Sbjct 916 ACGGCAGCTTTATTATGAAACCCACTTTAAGGTGACACTGATACTGGTACTCAAACACTG
Query 240 GTGACAGGCTAAGGATGCCCTTCAGGTACCCCGAGGTAACACGCGACACTCGGGATCTGA
Sbjct 976 GCGACAGGCTAAGGATGCCCTTCAGGTACCCCGAGGTAACACGCGACACTCGGGATCTGA
Query 300 GAAGGGGACTGGGGCTTCTATAAAAGTGCCCAGTTTAAAAAGCTTCTATGCCTGGATAGG
Sbjct 1036 GAAGGGGACTGGGGCTTCTATAAAAGTGCCCAGTTTAAAAAGCTTCTATGCCTGGATAGG
Query 360 CGACCGGAGGCCGGCGCCTTTCCTTTGACCACTACTGTTTACATGAACATGACCGACTGC
Sbjct 1096 CGACCGGAGGCCGGCGCCTTTCCTTTGACCACTACTGTTTACATGAACATGACTGACTGC
Query 420 TTTATCGCTTTGTTGTACGCCATCAGGGAGATCAAAGCACGACTTCTTCTACGGACACAA
Sbjct 1156 TTTATCGCTTTGTTGTACGCCATCAGAGAGATCAAAGCACGACTTCTTCTACGGACACAA
Query 480 GAGAAA 485
Sbjct 1216 GAGAAA 1221

Region 2: 4072 to 4267GenBankGraphicsNext MatchPreviousMatchFirst Match

340 bits(184) 1e-89 192/196(98%) 0/196(0%) Plus/Plus
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Sbjct 4072 GGACCCGACTTTAACCGGTTGGTTACCGCGTTTGAGGAATTGGCCACTGGGGTGAAGGCT
Query 605 ATCAGGACCGGTCTCGACGAGGCCAAGCCCTGGTACAAGCTCATCAAACTCCTAAGCCGC
Sbjct 4132 ATCAGGACCGGTCTCGACGAGGCCAAGCCCTGGTACAAGCTCATCAAACTCCTAAGCCGC
Query 665 CTGTCACGTATGGCCGCTGTAGCAGCACGGTCCAAGGACCCACTCCTTGTGGCCGTCATG
Sbjct 4192 CTGTCATGTATGGCCGCTGTAGCAGCACGGTCCAAGGACCCAGTCCTTGTGGCCATCATG
Query 725 CTGGCTGATACCGGCC 740
Sbjct 4252 CTGGCTGATACCGGCC 4267

Region 3: 1740 to 1841 GenBankGraphics Next MatchPreviousMatchFirst Match

156 bits(84) 6e-34 96/102(94%) 0/102(0%) Plus/Plus
Query 443 GGGAGCCGGGCAATCCAGCCCGGCAACCGGGTCGCAGAACCAGTCAGGCAACACTGGAAG
Sbjct 1740 GGGAGCTGGGCAATCTAGCCCGGCAACCGGATCGCAGAACCAGTCAGGCAACACCGGTAG 1
Query 503 CATCATTAACAACTACTACATGCAGCAATACCAGAATTCCAT 544
Sbjct 1800 CATCATCAACAACTACTACATGCAGCAATACCAGAATTCCAT 1841

Fig. 3 — Sequence alignment of the 786 nucleotides corresponding to IRES-2B (-L) in the replicon with the available sequence of FMDV - type Asia 1 isolate IND 81-86, complete genome (Sequence ID: gb|DQ989306.1|Length: 8227Number of Matches: 3).

Characterization of the Viruses Produced

The lysates from the FLO and FLOΔL infected cell monolayers were further studied for Lb protein expression by SDS-PAGE and immune blot using antiserum raised against *E. coli* expressed Lb protease. The lysate supernatants were collected 48 hr post infection and the ammonium sulphate precipitated proteins were analyzed by 15% SDS PAGE and immuoblot assay for the presence or absence of Lb protease in the infected cells. Positive colour reaction was observed with antiserum against Lb protease in the case of lysates from chimeric 'O' infected BHK cells but not in the case of 'O'-Lb infected cells (data not shown) indicating there was no Lb expression in Lb deleted virus. However, Lb deleted virus showed infectivity both in wild BHK cells and BHK cells expressing T7 polymerase, but the efficiency of replication was lesser than 'O' in BHK cells. This indicates that the Lb deleted virus is less virulent.

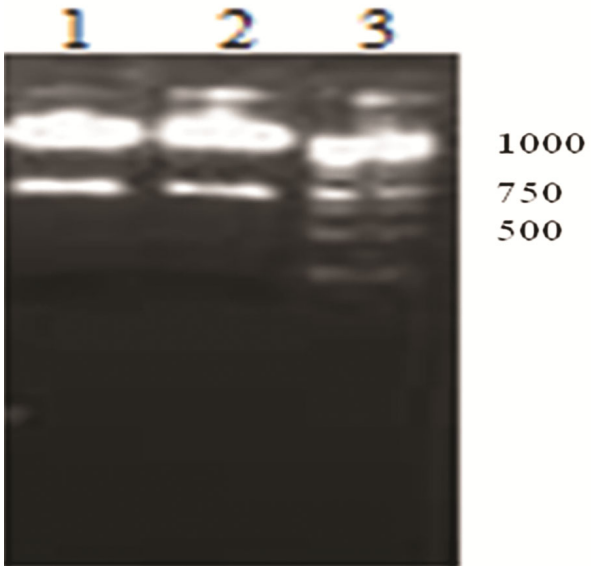

Fig. 4 — Release of GFP insert from Lb deleted replicons pFLA(v)-L (Lane 1, 2) Lane 3: 1 Kb ladder.

Further, the virus replication in the RNA transfected cells were confirmed by the presence of virus specific 5' L-UTR amplification from the cDNA prepared with the primer corresponding to 3' end of the viral RNA i.e 3'UTR (R) primer from the RNA isolated from transfected cells 48 hr post infection. One µg of total RNA was used for amplification studies.

As seen in the Figure 8, (lanes 1 - 3) below all the transcripts showed the amplification of around 750 bp fragment from all the cDNAs. Intense amplification was seen with full length plasmid DNA which was

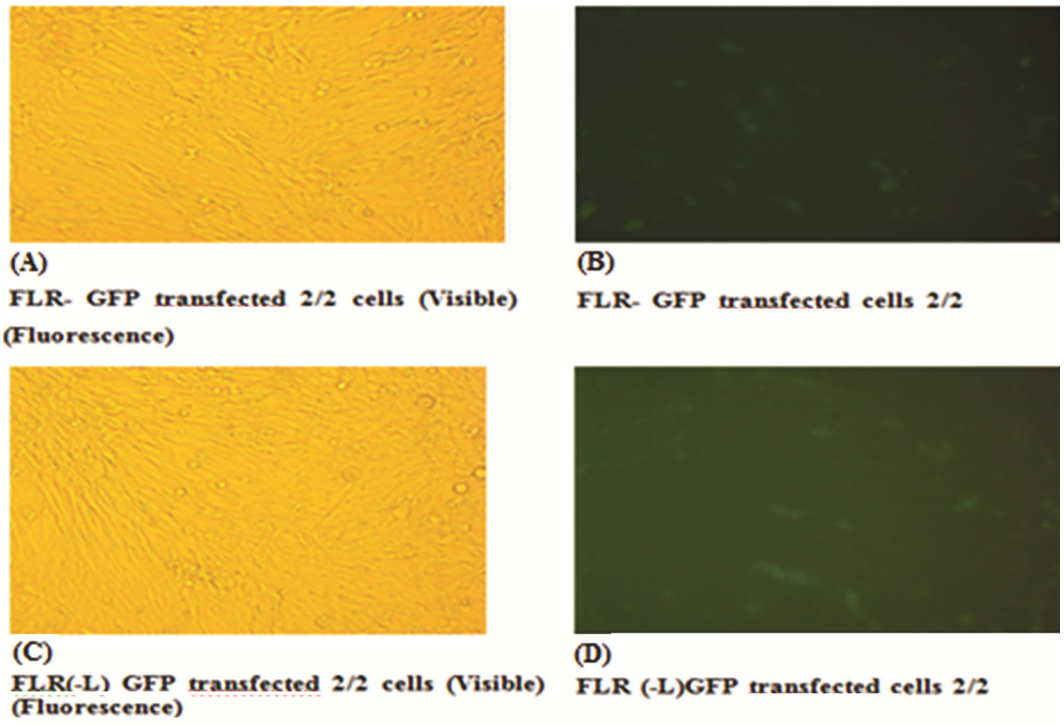

Fig. 5 — A: GFP expression in BHK cells, B: Lb undeleted, C: the transfected cells visualized under visible light and D: Lb deleted and GFP inserted replicons as visualized under fluorescence.

used as positive control (lane 3). In the case of chimeric biosynthetic viruses (lanes 1, 2) there was a marked difference in the intensity of the amplified products. It was less in the case of FLOΔL indicating the efficiency is less which may be due to the absence of Lb protease which is needed for virulence. Hence this can be evaluated as an attenuated vaccine in animal model.

Discussion

Previous strategy to generate attenuated viruses for vaccine development is by serially passaging the virus in non permissive host systems and selecting the mutant viruses that are naturally selected. The mutations that occur in natural selection are generally of point mutation- type or limited deletions that can affect the virus replication to a limited extent but to a great extent viral pathogenesis to the host system. Such natural mutations are highly prone to reversion making the virus to be virulent thereby causing disease outbreaks in the vaccinated animal.

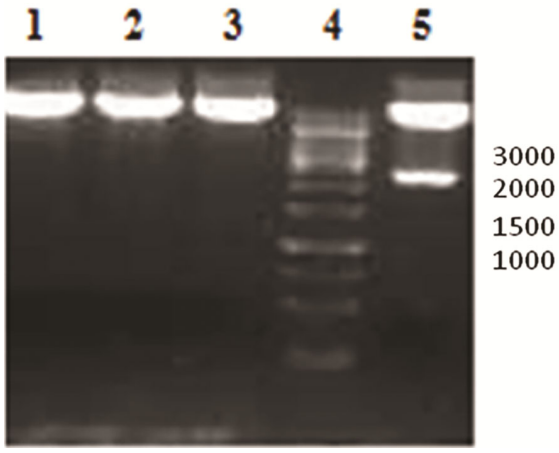

Fig. 6 — *Bam*HI digested pBFLOΔLb with and without P1-2A insert. Lane 1, 2, 3: construct without insert Lane 4: 1 kb ladder Lane 5: with P1-2A insert

In recent past several issues have come up, questioning their safety and therefore some countries like USA have stopped using such vaccine. Therefore, alternative approaches have been worked out which includes use of chimeric viruses where the gene encoding for immunogen is inserted into non host specific virus and use as vaccines, best examples are use of non host specific adenoviruses and some poultry viruses for preparation of vaccines against animal diseases. However, such approach is not found to be successful for the development of potent vaccines against some of the diseases where immunogen expression alone is not sufficient. Foot and mouth disease of cloven footed animals is one of such disease concern being aimed for global eradication. Natural selection of attenuated FMDV through passage in mouse brain was found to be less stable as safe vaccine. Presently, chemically inactivated FMDV of three anti genically different serotypes are used for mass vaccination to control the disease. However, this has several issues like poor immunogenicity and short duration of immune response needing repeated vaccination, risk of escape of the pathogen from manufacturing units and presence of un-inactivated virus in the vaccine causing disease outbreaks in vaccinated animals. Hence, global eradication of FMD became a challenging task, resulting in constant search for an alternative vaccine development. One of the sure shot method is to develop live attenuated FMDVs of all the serotypes with stable mutation/s and use for mass vaccination. This may be possible only by making known non-immunogenic gene deletion in the laboratory and use as attenuated vaccine. The gene deleted viruses can be used as marker vaccine as the detection of antibodies against deleted gene product will help to differentiate the vaccinated animals from the infected.

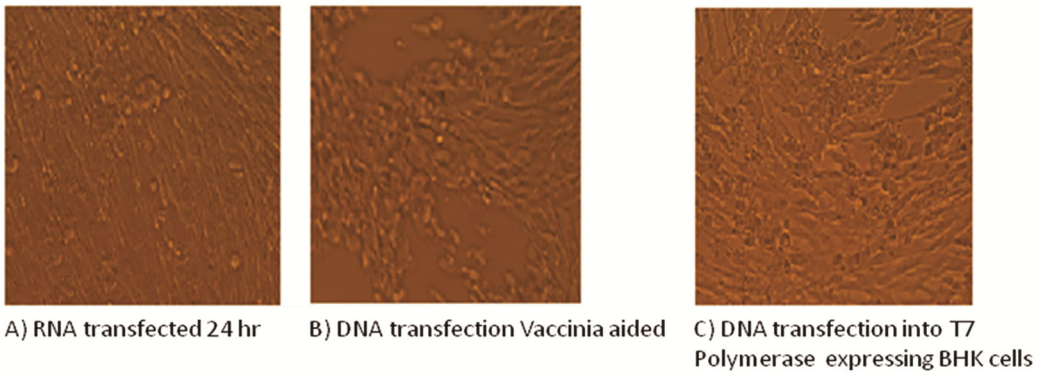

Fig. 7 — Infectivity of the transcripts of pBFLO and pBFLOΔLb to BHK21 cells.

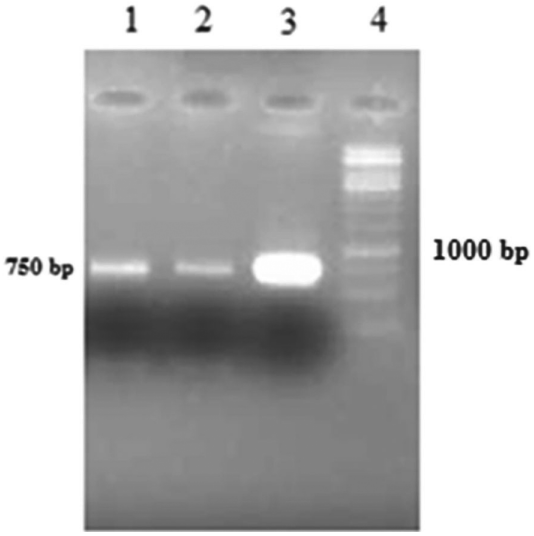

Fig. 8 — Amplification of 5' UTR region from the cDNA of the RNA from transfected 2/2 cells.
Lane 1: Lb deleted FLO, Lane 2: Lb deleted FLO, Lane 3: Positive control full length FLO, Lane 4: 1000 bp Ladder

Numerous studies have been conducted with genetically modified serotype A FMDVs lacking L protease which was shown to be infectious but grow more slowly in cell culture and is attenuated in both cattle and swine (Brown *et al,* 1996). Lb is considered to be the biologically functional protein following initiation of which results in production of live virus while the deletion of first AUG codon has no effect on viral replication. A serotype A12 virus that lacked the L-coding region (leaderless) was constructed. This virus replicated in BHK-21 cells but did not cause disease in cattle or swine (Piccone *et al*, 1995). It greatly reduced pathogenicity in susceptible animals, leaderless virus was examined as a live attenuated vaccine candidate, induced neutralizing antibody response in both the species. Taken together, our results and those of Brown and Piccone *et al*. suggest that the deletion of Lb^{Pro} approach can be used for the production of replication defective FMDV and subsequent studies on their use as attenuated vaccines.

The L-protease gene responsible for pathogenesis was selected for deletion studies and deletion was successfully carried out. Since, this approach is to be followed for all vaccine strains to be used as polyvalent vaccine, we have made FMDV replicon with Asia 1 serotype and used the replicon for deletion . This would help development of polyvalent live attenuated vaccine. There was reduction in virulence in the case of Lb deleted RNA as seen *in vitro* studies using BHK cell monolayers indicating the Lb deleted virus may be used as an attenuated virus vaccine.

Towards the development of companion tests for marker vaccines, and for evaluating the gene deleted viruses *in vitro* for replication efficiency, we have cloned the deleted genes in expression vector, expressed the proteins in *E. coli*, raised antisera (Sarkar *et al*, 2017) in experimental animals and used. The viruses need to be further evaluated for attenuation and immunogenicity in homologous host.

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