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Full Length Research Paper

Construction of genome-length cDNA for foot-and-mouth disease virus serotype Asia 1 IND 63/72 vaccine strain

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Foot-and-Mouth Disease (FMD) is a highly contagious and economically important viral disease of cloven-hoofed animals. The virus (FMDV) belongs to the genus *Apthovirus* of the family *Picornaviridae*. The FMDV genome consists of a positive sense ssRNA of about 8500 nt encoding a single open reading frame (ORF) which later processed into multiple viral proteins. Although FMDV replication resembles those of other picorna viruses, there are notable differences like broad host range and several unique genetic features. On the Asian continent, FMDV serotype Asia 1 plays a second major role in causing outbreaks after serotype O. The Asia 1 virus displays unique characteristics in its stability, replication kinetics and plaque morphology. In order to study these characteristics we have constructed a genome-length cDNA clone of FMDV Asia 1 vaccine strain IND 63/72. The functionality of the cDNA was checked in BHK 21 cells and it did not yield any viable virus particle. The genome-length cDNA contained a single ORF of 6902 nucleotides terminating at a UAA codon 95 bases from the 3' poly (A) tail sequence. The 8167 base pair sequence and the deduced amino acid sequence (2330 aa) were compared with the published FMDV sequence of Chinese strain YNBS/58 showed 5.3% variation at amino acid level.

Key words: Foot-and-mouth disease virus, Asia 1, *Apthovirus*, genome-length cDNA, *In vitro* transcription and translation.

INTRODUCTION

Foot-and-Mouth Disease (FMD) is the most contagious viral disease of cloven-hoofed wild and domesticated animals and the earliest recorded account was made by Fracastorius in 1546 (Brooksby, 1982). It was the first animal disease demonstrated to be caused by a filterable agent in 1897 by Loeffler and Frosch. The agent has later been identified as an RNA virus (FMDV), belonging to the genus *Apthovirus of* the family *Picornaviridae* (Franki et al., 1991).

The disease is characterized by high morbidity in adults and low mortality in young animals with typical clinical signs of oral and pedal vesicles which can result in significant decline in production of dairy and meat products. The disease is enzootic in many parts of the world including Asia, Africa and South America and the incidence is very low in many of the European countries (Domingo et al., 1990). In India, the disease is endemic and outbreaks occur throughout the year and in all parts of the country. Majority of the outbreaks are caused by type "O", followed by Asia 1, A and C. Type C is the least prevalent and has not been recorded since 1996. The disease is controlled mainly by regular vaccination using inactivated virus as vaccine.

The FMD virion is a non-enveloped icosahedron of 25-30 nm in diameter and the genome consists of an infectious, linear single stranded, positive sense RNA of 8.5 kb. The RNA is uncapped however, virus coded protein VP_g (3B trimer, 3 kDa) is covalently bound to it at 5' end (Grubman, 1980) and 3' end has a poly (A) tail.

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The genome has an ORF flanked on either ends by untranslated regions (UTRs). The 5'UTR of 1300 nucleotides (nts) comprises S fragment (400 nts) followed by poly(C) tract (80 - 200 nts) (Harris and Brown, 1977; De la Torre et al., 1988) and an L fragment (700 nts) which has an Internal Ribosome Entry site (IRES) 435 nts upstream of the start codon. It has a major role in initiation of viral translation in a cap independent fashion (Belsham, 1993; Tratschin et al., 1995). The single viral ORF is of 6996 nts, has two initiation sites both in frame with strong initiation from the downstream start codon and codes for a polyprotein (Fross et al., 1984). Following the ORF is a short 3' UTR (190 nts) and a poly (A) tract (10 - 100 nts) (Chatarjee et al., 1976). The 3' UTR is believed to contain major cis-acting specific sequences signal required for negative-strand RNA synthesis, and is essential to complete a full replication cycle (Saiz et al., 2001). The single ORF consists of 3 polyprotein coding regions, from the 5' end to the 3' end, P1, P2, and P3. The P1 region consists of 1A, 1B, 1C, 1D genes, which codes for the structural proteins VP4, VP2, VP3, and VP1, respectively (Rueckert and Wimmer, 1985). P2 and P3 regions encode non-structural proteins 2A, 2B, 2C and 3A, 3B, 3C, 3D, respectively. The 2A region is highly conserved in all the serotypes (Vakharia et al., 1987). 2C is probably involved in virion RNA synthesis (Saunders et al., 1985). 3B encodes VPg (Fross and Schaller, 1982) and protein 3D functions as the viral RNA polymerase, also known as the Virus Infection Associated Antigen (VIAA) (Polatnick, 1980) used for differentiating infected and vaccinated animals.

The potential of investigating RNA viruses by establishing infectious genome-length cDNA clones has greatly increased after the discovery of the reverse transcriptase enzyme. The availability of cDNA clones makes it possible to analyze and modify genomes at molecular level and helped in the study of gene organization and expression, host-virus interactions, design of antiviral strategies, trans-complementation studies of segmented RNA viruses, development of new viral vectors and for the development of new generation vaccine (Boyer and Haenni, 1994). At present, the infectious cDNA clones of FMDV have been established for O1K from cattle (Zibert et al., 1990), OH99 from swine (Liu et al., 2004), for A12 (Rieder et al., 1993) and for SAT2 vaccine strain ZIM/7/83 (van Rensburg et al., 2004).

Asia 1 remains endemic in India, parts of China, Bangladesh, Israel, Malaysia, Myanmar, Thailand, Laos and Cambodia (Kitching, 1999). The Asia 1 virus is unique in terms of its stability, replication kinetics and plaque morphology (Rajdan et al., 1996). The molecular basis of these characteristics is not understood. Preliminary studies on 5' untranslated region (UTR) (Tratschin et al 1995) showed that variation in internal ribosomal entry site (IRES) might be responsible for variability in host specificity. However, these characteristics can be better understood through infective cDNA clone. So the the present study was undertaken to develop infectious genome-length cDNA clone of FMDV serotype Asia 1 vaccine strain IND63/72 to study its characteristics.

MATERIALS AND METHODS

Cell lines and viruses

Baby hamster kidney (BHK) cells, strain 21, clone 13 maintained in Glasgow Minimal Essential Medium (GMEM) containing 10% fetal bovine serum (FCS) and 10% tryptose phosphate broth were used. Foot and mouth disease virus vaccine strain serotype Asia-1 (IND 63/72) maintained at FMD centre of IVRI, Bangalore, India was once plaque purified passaged once in BHK-21 cells and used as a source of virus.

RNA extraction and cDNA synthesis

The viral RNA extracted directly from infected cell culture supernatant at passage level 6 in BHK21 cell was used for cDNA synthesis using Trizol reagent as per Manufacturer's (Gibco BRL) protocol and used as a template for cDNA synthesis. Superscript (Moloney murine leukemia virus (MMLV) reverse transcriptase, Life Technologies, USA) and gene specific primers corresponding to various regions of the RNA genome were used for the reverse transcription reactions, which were carried out for 90 min. at 37°C. The primer sequences used for the synthesis of cDNA were shown in Table 1.

PCR amplification, construction of genome-length cDNA clone and sequencing

The genome-length cDNA for FMDV serotype Asia 1 (IND63/72) was constructed in the pBSKS+ vector by linking the PCR amplified overlapping fragments. The different regions of the Asia 1 virus were obtained through PCR amplification from cDNA using EXT and Dynazyme DNA polymerase (Finnzyme). The reactions were carried out in the presence of oligonucleotides designed to contain restriction enzyme sites that would facilitate the linking of fragments. The sequences of the primers used for amplification and linking were shown in Table 2. First, the LUTR to 2A fragment was amplified and cloned into pBSKS+ vector in the *Hind*III and *Not*I RE sites (pKSLUTR-2A).

The VP1-2A fragment was amplified and cloned sequentially first into pRSETA (*Bam*HI and *Bgl*II) and the fragment released with *Bam*HI and *Eco*RI and cloned into pcDNA3.1+ vector using same sites. Then the fragment was sub-cloned into pBSKS+ vector in *Hind*III and *Eco*RI sites (pKSVP12B). The fragment containing LUTR to 2B was obtained by releasing the LUTR-2A fragment in pKSLUTR-2A with *Hind*III and *Nhe*I sites and cloned into the pBSKS+ vector containing VP1-2A fragment and the resulting plasmid named as pKSLUTR2B. The 2BC fragment was amplified using forward and reverse primer containing *Bgl*II and *Eco*RI RE sites respectively and cloned into pKSVP12B, to get the fragment from VP1 to 2BC with partial 3A region (pKSVP12BC3A).

The 3D fragment was amplified and cloned into *Nhe*I and *Not*I sites of pcDNA3.1+ vector. This fragment was released with *Nhe*I and *Pme*I and subcloned into *Nhe*I and *Eco*RV sites of pBR322 vector. Further, the fragment released *Nhe*I and *Hind*III and cloned into pKSVP12B using same RE sites and the resulting plasmid named as pKSVP12B-3D. The 3ABC fragment was amplified and cloned into *Eco*RI and *Nhe*I sites of pKSVP12B-3D plasmid to get pKS3ABCD. The 3ABCD fragment was released from the plasmid pKS3ABCD with *Bam*HI and *Eco*RI and subcloned into pKSVP12BC3A to get the fragment containing VP1 to 3D region

Table 1. Primers used for first strand complementary DNA synthesis of FMDV genome.

S/No.	Primer	Sequence	Targeted FMDV gene
1	PrepolyCR(SmaI)	5'GGGCCCGGGGGGGGGTGAAAGGC3'	SUTR-Poly(C) tract
2	LedRm	5'TCGGACGGATCCGGCGTCCA3'	LUTR
3	2BR	5'GCCGTTCTTGAGAATGGC3'	L-protease, P1-2A, 2B
4	3CRNot	5'GGGCGGCCGCCTCGTGTTGTGGTTCAGG3'	2C
5	OligodT OligodT(SexA)	5'TTTTTTTTTTTTTTTTTT3' 5'ACACCAGGTTTTTTTTTTTTTTTTTT3'	3AB, 3C, 3D
6	cDNA(PolyA)NH2-P1	5'GGCCATGGTACGTAGGATCCGGAAAAAAAAAA3'	3'UTR

Table 2. Primers used for amplification of overlapping fragments of FMDV Asia 1 IND 63/72 genome.

S. No	Gene amplified	Primer sequence
1	T7-Small UTR	STJD+(F): 5'GCGAGTACTGTAATACGACTCACTATAGGTTGAAAGGGGGCGCTAG3' PrepolyCR(Sma)(R): 5'GGGCCCGGGGGGGGGTGAAAGGC3'
2	Large UTR	5.7UTL(Apa)(F): 5'CCCGGGCCCCCCCCTTTTACCGTCGTTCCCGAC3' 5.7LUTR(R): 5'CCCGGGCCCCCCCCTTTTACCGTCGTTCCCGAC3'
3	Lpro-P12A	Seq2(F): 5'GAGGTAACACGCGACACT3' 2AR(NotI)(R): 5'GCTAGTGCGGCCGCGAAGGGCCCAGGGTTGGACTC3'
4	Partial VP1-2B	SV1(F): 5'GGGCGGGGATCCGATGGCGCCACACCGTGTGTTGGC3' 2BR(R): 5'GCCGTTCTTGAGAATGGC3'
5	2BC-partial 3A	P2L(F): 5'GAGTCCAACCCTGGGCCCTT3' 3ARM(R): 5'CCATCCCCTCAAAGAATTCAAT3'
6	3ABC-partial3D	3ALn(F): 5'GGTGATTGACCGGGTTGAG3' 3CR(Nhe)(R): 5'AACGGTGGGTGCTAGCTTGGT3'
7	3D-3'UTR-19A	3DL(Nhe)(F): 5'ACCAAGCTAGCACCCACCGT3' OligoT(SexAI)(R): 5'ACACCAGGTTTTTTTTTTTTTTTTTT3'
8	3D	3DL(Nhe)(F): 5'ACCAAGCTAGCACCCACCGT3' 3DR(Not)(R): 5'GCTAGTGCGGCCGCTTATGCGTCACCGCACACGG3'

(F) – Forward primer; (R) – Reverse primer.

(pKSVP13D). The fragment VP1-3D was released from the plasmid pKSVP13D using *Bgl*II and *Not*I and subcloned into pKSLUTR2B to get the fragment from LUTR to 3D (pKSLUTR3D). The 3'UTR containing 19 A residues at 3' end were amplified along with 3D and cloned into pcDNA3.1+ vector using *Nhe*I and *Eco*RI RE sites (pc3D-3'UTR). Then the 3'UTR region was released from pc3D-3'UTR using *Pst*I and *Not*I and subcloned into pKSLUTR3D to get the fragment from LUTR to 3'UTR (pKSLUTR-3'UTR).

The 5' oligonucleotide primer containing the T7 promoter and 17 nucleotides of IND63/72 sequence (*Bam*HI) and 3' primer containing *Sma*I was used to amplify the short-untranslated region (S-UTR). The amplified fragment was blunt ended and inserted into pBSKS+ vector (pKSSUTR). The L-UTR fragment was amplified with the *Apa*I site at 5' end, was digested and blunt ended with T4 DNA polymerase followed by digestion with *Eco*RI at 3' end. This fragment was cloned into pKSSUTR in the *Sma*I and *Eco*RI sites to get the plasmid pKS5'UTR. The plasmid pKS5'UTR was digested with *Bam*HI and *Xba*I and subcloned into the plasmid digested with same RE sites and gel purified vector backbone. The resulting plasmid pKSFAs contained the genome-length cDNA of FMDV serotype Asia 1 (IND63/72). The complete sequence of the assembled genome-length cDNA was submitted to Gene bank

with accession no. AY304994 and AY319417.

In vitro transcription

The full-length pFAS plasmid was linearized with *Not* I and extracted with phenol/chloroform, ethanol precipitated and redissolved in RNAse free water. Typically, *in vitro* transcription was performed with 0.5 – 1 µg of linearized full-length cDNA template in a 20 µl reaction using T7 RNA polymerase (Promega) according to the manufacturer's instruction. The transcribed RNA was treated with 1 U of RNAse free RQ1 DNAse (Promega) at 37°C for 30 min. to remove the linearized DNA template. The size and quality of the *in vitro* transcribed RNA was checked by electrophorese in 1.2% agarose gel in Tris-borate-EDTA buffer in the presence of ethidium bromide.

Transfection experiments in BHK21 cells

Transfections of the *in vitro* transcribed RNAs were performed on BHK 21 cells by using Lipofectamine 2000 (Invitrogen). One day before transfection, 0.5–2 × 10^5 cells in 2 ml of growth medium were seeded in the wells of 6 well plate. At 90 – 95% confluency, the cells were transfected with the RNA and transfection mixture prepared in Opti-MEM medium (Invitrogen) and incubated at 37°C with 5% CO_2 for 4 – 6 h. At the end of incubation, the cells were incubated with fresh Eagle's Minimum Essential Media (EMEM) containing 2% sera for 48 h. The cells were observed for cytopathic effect (CPE) for 48 h and if the CPE is not clear during incubation, either cell supernatant or freeze-thawed cell lysates were blind passaged twice on fresh BHK 21 monolayer cells.

In vitro translation

In vitro translation of the linearized full-length cDNA was performed in rabbit reticulocytes using TNT coupled transcription and translation system (Promega). About 2 µg of DNA was added to an aliquot of the master mix in a 25 µl reaction volume and incubated for 90 min. at 30°C. Luciferase from Renilla and firefly were included in the experiment as a positive control. Synthesized proteins were then analyzed by SDS-PAGE and autoradiography.

RESULTS

Assembly of genome-length cDNA constructs

Sequencing of the overlapping fragments showed unique *NheI* site in the P1-2A gene. Likewise, the *BglII* site identified in the 2B region was used to link the upstream and downstream half of the genome. There is a conserved *PstI* site in the 3D polymerase gene. The restriction site, *XbaI* in the LUTR was identified as unique site. This has been found to be unique for all the FMDV serotypes.

The amplified and cloned fragments were sequenced and confirmed for integrity before being used for final construct. The final construct in pBSKS+ vector was obtained by linking the overlapping fragments and the whole genome was sequenced to confirm the size and coding region of each gene (accession nos. AY304994 and AY319417).

In vitro transcription and infectivity study of RNA into BHK21 cells

RNA was *in vitro* transcribed from the *Not* I linearized plasmid carrying genome-length cDNA (pKSFAs) with T7 RNA polymerase and the quality of the RNA was analysed by gel electrophoresis, which showed the intact and expected size of the transcribed RNA. This RNA contained two non-genomic G residues (added to increase T7 promoter efficiency) at 5' end, a poly(C) tract of 12 nucleotides and 23 non-genomic bases after a 19 base poly (A) tail at 3' end.

To study the infectivity of the genome-length cDNA, the *in vitro* transcribed RNA was transfected into BHK 21 cells using the transfection reagent Lipofectamine 2000 (invitrogen). Apparent CPE is generally observed in case of RNA purified from wild type virus after 24 h, but in the case of *in vitro* transcribed RNA CPE-like appearance was observed after 48 h of incubation (Figure 1A and B). The *in vitro* transcribed RNA-transfected cell supernatant and the freeze thawed cell lysate was passaged twice through BHK 21 cell monolayers. Though initially cells showed rounding and CPE-like appearance, after 48 h the cells recovered and there was no lysis of even after 72 – 96 h of incubation.

Coupled transcription and translation

In order to check the translation and processing of the virus, specific proteins *Not*I linearized plasmid (2 µg) was subjected for coupled transcription and translation in rabbit reticulocytes. After the incubation of sample along with positive control at 30ºC, the translated proteins were run in SDS-PAGE and autoradiographed. There was a ladder of proteins observed in the sample lane. It indicated that complete FMDV genome was expressed and processed by viral proteases (like L protease, 2C protease and 3C protease).

So the expressed proteins were cleaved, processed and was seen as multiple bands in the gel (Figure 2, Lane 1). In positive control there was no such ladder, only two bands corresponding to the expressed proteins Firefly and Renilla luciferase, 31 and 65 KDa respectively were noticed (Figure 2, Lane 2). It indicated the integrity, stability and completeness of the FMDV Asia-1 full-length construct (pKSFAs).

DISCUSSION

Foot-and-mouth disease (FMD), a highly contagious and economically important disease is caused by picornavirus which mainly affects wild and domestic cloven-hoofed animals. FMDV populations are genetically heterogeneous and exhibit an important potential for variation and adaptation (Domingo et al., 2003). Although FMDV replication resembles those of other picorna viruses,

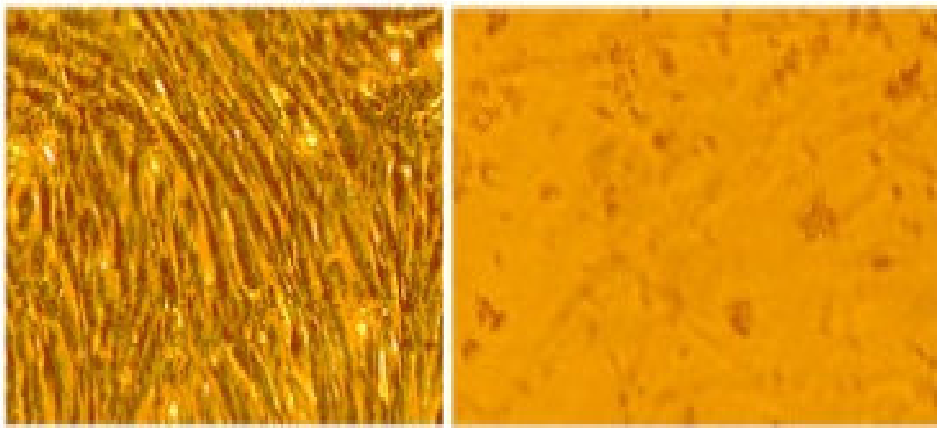

Figure 1. Infectivity of RNA transcript from full length cDNA of Asia 63/72 in BHK 21 cell monolayer cells (A) Mock infected cells (B) Infected with RNA transcript from full length cDNA.

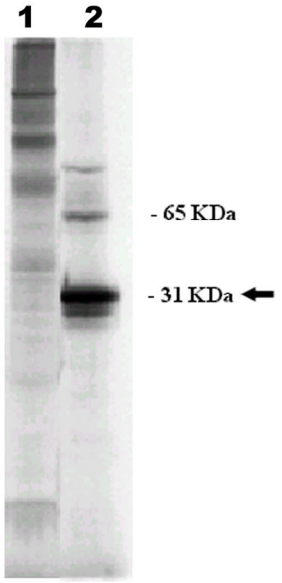

Figure 2. Coupled transcription and translation of Not I linearized genome-length cDNA in rabbit reticulocytes. Lane 1 - Proteins from FMDV serotype Asia-1 genome-length construct. Lane 2 - Proteins from firefly and renilla luciferase positive control.

there are notable differences like broad host range and several unique genetic features. Apart from this, the mechanisms involved in FMDV virulence and pathogenicity are not well known (Mason et al., 2003; Jackson et al., 2003). To elucidate these mechanisms, the genome-length infective cDNA of FMDV and its recovery of infectious virus in the susceptible cell lines *in vitro* is an important tool. Infectious cDNA clones of O1K (Zibert et al., 1990), A12 (Rieder et al., 1993), OH99 (Liu et al., 2004) and SAT2 (ZIM/7/83) (van Rensburg et al., 2004) have been constructed and characterized. On the Asian continent, FMDV serotype Asia 1 plays a second major role in causing outbreaks after serotype O. The Asia 1 virus displays unique characteristics in its stability, replication kinetics and plaque morphology (Rajdan et al., 1996). In order to study these characteristics we have constructed a genome-length clone of FMDV Asia 1 vaccine strain IND 63/72.

For the synthesis of genome-length cDNA of Asia 1, we have used amplification of multiple fragments, cloning and linking of overlapping fragments strategies. The primers used in this study were initially designed from the published sequences of O1K (Forss et al., 1984; Accession No. X00871), A12 (Robertson et al., 1985; Accession No. M10975) and Asia 1 5'UTR (Accession No. Y17973), L-protease and non-structural proteins (2C, 3C and 3D) (Genbank Accession No. AF207520, AF207524, AF207525 and AF207521). *NheI* site which was found as unique in P1-2A of Asia 163/72 was used to link the fragments of L-Protease-P1-2A and 2B. One more *NheI* site was introduced into 3D gene without changing the amino acid sequences of gene, and was used for linking the 3ABC to 3D. The conserved Pst 1 was used to link the 3'UTR sequences to the rest of the genome. The unique Xba 1 site was used to link the fragments of small and large 5' UTR with the rest of the genome. The plasmid vectors pBSKS+, pRSETA, pBR322, pcDNA3.1+ and pUC19 were chosen to use the available restriction enzyme sites for the cloning of different overlapping fragments of the IND 63/72 viral genome. The final genome-length construct was made in the pBSKS+ vector, downstream of T7 promoter which

was introduced through 5'UTR(S) forward primer. The proof-reading DNA polymerase (pfu DNA polymerase from Fermentas Life Sciences) was used for the synthesis of genome fragments, in order to minimize the PCR mutation. Unique restriction enzyme sites present either in the viral genome or in the vector were introduced in the forward and reverse primers and these amplified fragments were linked to get the genome-length cDNA.

The T7 promoter sequence was introduced at the 5' end to the start of viral genome (5'-UUGAAA-3'), which had two extra G nucleotides (non-viral sequence) added to increase the efficiency of the promoter. It is generally found that the presence of non-viral nucleotides at the 5' end of viral transcripts strongly reduces infectivity (Heaton et al., 1989; Dore et al., 1990). But it had already shown that the extra two G residues did not affect the infectivity of OH99 cDNA (Liu et al., 2004). The 5'UTR of cardioviruses and aphthoviruses contains a homopolymeric Poly (C) tract of unknown function. The mechanisms leading to the size variation of the homopolymer is not clear (Costa et al., 1984). Zibert et al. (1990) considered that poly (C) tract is required for FMDV to replicate in tissue culture. But infectious cDNA clones of FMDV type A12 which contained poly (C) tract of 2 – 35 nucleotides were equally virulent in mice (Rieder et al., 1993). In this study we have constructed the Asia 1 IND 63/72 cDNA containing 12 C residues in the poly (C) tract.

The 3' UTR and poly (A) tail at the 3' end of the transcripts may also play an important role in the infectivity by permitting the hybridization of the 3B-pUpU primer to allow synthesis of the negative sense copy of the viral genome (Barton et al., 1995; Saiz et al., 2001). But the minimum length of the adenosine residue required for infectivity of RNA is unknown. In our genome-length cDNA we have included 19 A residues at the end of 3' UTR. Further, we have included the *Not*I site to linearize the cDNA before *in vitro* transcription to minimize the non-viral sequences. When the sequence of the 3' UTR was compared with the other serotype sequences, it was found to be conserved among FMDV serotypes.

The complete genome was characterized at DNA level for the presence of UTR and coding sequences, unique restriction enzymes, open reading frame and at protein level for expression of structural and non-structural proteins. The fragments linked were sequenced before and after linking for confirming the junctions and unique RE sites at the linking region. The 8167 base pairs sequence and the deduced amino acid sequence (2330 aa) were compared with published FMDV sequence which showed 5.3% variation at amino acid level with Chinese strain YNBS/58. The 5' end of the genome upstream from homo polymeric poly (C) tract (S fragment) was 370 nucleotides in length and the remainder of the genome (L-fragment), including the poly (A) tail was 7797 nucleotides. The L-protease fragment contained a single ORF of 6902 nucleotides terminating at a UAA codon 95 bases from the 3' poly (A) sequence.

The *in vitro* transcribed RNAs were synthesized from genome-length cDNA constructs using T7 RNA polymerase. The purified *in vitro* transcripts were transfected into BHK21 cell monolayers and the cells were observed for 48 h. There was no specific cytopathic effect of FMDV observed. Initial changes could be observed after two blind passages of transfected cell supernatant and freeze-thawed cell lysates in fresh BHK21 cells; however, the cells recovered showed no lysis indicating that the virus was not stable or cDNA was not infective. However the coupled transcription and translation of the genome-length cDNA showed the production of FMDV specific proteins.

Although obtaining genome-length cDNA clones and/or the corresponding transcripts is a crucial step, it does not necessarily ensure biological activity. Infectivity of transcripts is variable and can in some cases reach 100% as compared to infectivity of wild type virion RNAs. To achieve successful infection, viral transcripts must interact with viral encoded proteins, most particularly with the viral replicase and with host cell components such as the translation machinery. Therefore, the structure of viral transcripts has to mimic that of virion RNA as closely as possible. Several parameters have a dramatic influence on the infectivity of viral transcripts: the heterogeneity of transcript population, the sequence at the 5' and 3' ends and its compatibility with the sequence of the coding region. One may presume the compatibility of 5'UTR with internal sequence may be of primary importance as the 5'UTR used here was amplified at different time point though from single plaque virus. We do not have any data to prove this hypothesis. Our recent studies show that replacement of 5' UTR along with Poly(C) track amplified at a stretch from single viral RNA helps to achieve infectivity (Unpublished data). The heterogeneity of transcript size is responsible for competition between incomplete non-replicable viral copies and genome-length transcripts for interaction with viral and/or host factors involved in the replication process (Hamilton and Baulcombe, 1989). Because of the relatively poor fidelity of the RNA synthesizing enzymes used, in *in vitro* experiments point mutations are expected, especially with long viral genomes. It was reported that alteration of viral sequences could result from the *in vitro* transcription step since sequence dependence of T7 and SP6 RNA polymerase fidelity has been observed (Kuhn et al., 1990). Further, the final genome-length cDNA clone can be the result of faithful reverse transcription and amplification of an initial virion RNA which itself may be of mutated version, unable to replicate and which would probably be eliminated in the next round of viral replication. As a consequence, this cDNA clone or the corresponding *in vitro* transcript would not be infectious. Error prone *in vitro* transcription coupled with low fidelity of viral RNA dependent RNA polymerases (Domingo et al., 2003) may be responsible for failure in the production of infective

virus. The production of non-infectious transcripts has been reported and some showed the possibility of restoring infectivity by exchanging a specific region of the cDNA with a fragment corresponding to the same region but replaced from independent cDNA clone (Boyer and Haenni, 1994).

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