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A strategy to develop disabled infectious single-cycle (DISC) foot and mouth disease virus by 3B3 gene deletion using the infective cDNA copy of the genome

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Disabled infectious single-cycle (DISC) virus is in between attenuated and inactivated. When used as a vaccine DISC virus behaves like inactivated virus as it cannot further multiply in the vaccinated individual after one cycle of replication. When infected to permissive cells expressing virus specific protein which has been deleted from viral genome, the virus replicates normally. The development of DISC virus involves the deletion of an open reading frame (ORF) coding for a key protein involved in the viral replication or viral capsid formation. Such virus, when injected in animals, can complete only one round of replication without producing a progeny virus. Here we report similar strategy followed for the production DISC foot and mouth disease virus (FMDV). We have selected 3B3 protein gene that expresses 3 copies of virus specific genome-linked protein needed for virus replication and deleted from the FMDV replicon carrying FMDV serotype Asia 1 backbone and inserted the same into baby hamster kidney 21 (BHK-21) cell genome. Upon transfection of the genetically modified BHK cells with RNA copy of the genetically modified cDNA, replication of the virus started in these cells. The DISC virus so developed can be a potent vaccine candidate for achieving robust and long duration of immune response against FMDV infection in bovine. The approach also took care in the development of serotype specific DISC viruses using single FMDV Asia 1 replicon. Vaccines based on DISC viruses may be superior in terms of immune response as compared to inactivated vaccines.

Keywords: Disabled infectious single cycle, 3B3 gene deleted replicon, BHK cells expressing 3B3, FMDV virulence, attenuation.

Introduction

Foot and mouth disease virus (FMDV), the positive sense 8.5 kb ribonucleic acid (RNA) virus has single open reading frame (ORF) which, upon infection is translated into a polyprotein of 250 kilodalton (kDa). The polyprotein is processed by the virus specific protein to yield 4 structural and 7 non-structural proteins and the non-structural proteins participate in the genome replication. Polyprotein is subjected to primary cleavage to yield the leader protein (L), the polyprotein 1 (P1), the polyprotein 2 (P2) and the polyprotein 3 (P3) from the N terminus to the C terminus. The P1 is further processed to generate the four viral structural proteins, 1A to 1D (VP1 - VP4). Following the P1 is the P2 that yields three viral NS proteins (2A - 2C) which are mostly needed in the host virus interactions. The P3 upon secondary cleavage yields 3 NS proteins (3A - 3C) of which 3B carries 3 copies of a polypeptide which is the genome linked protein (VPg) of 4 kDa that acts as a primer for RNA replication (both for positive and negative strand genome). Following the 3B is the 3C which is a protease involved in all cleavages of polyprotein and host specific initiation factor and 3D is the replicase.

Protein 3B (VPg) is covalently bound to the 5′ terminus of the genome and anti-genome and primes picorna virus RNA synthesis[1]. It exists in three similar but non-identical copies (3B1, 3B2 and 3B3), which are 23 - 24 amino acid (aa) long[2] although not all three copies of FMDV 3B are needed to maintain infectivity[3]. Uridylylation of the VPg is the first step in the replication of the picorna virus genome. The peptide is covalently linked to the viral RNA through the conserved Tyr3 residue by the action of viral RNA polymerase (3D pol). In this process, the viral 3D pol catalyzes the addition of two uridine monophosphate (UMP) molecules to the hydroxyl group of this Tyr3 using the *cis*-replicating element (bus/cre) in FMDV genome as template[4]. 3B3 is likely the most efficient

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substrate for 3D pol activity[5]. A reverse genetics study demonstrated that deletion of the 3B3 coding sequence exerts a deleterious effect on FMDV RNA replication, resulting in production of a non-infectious RNA transcript[6]. Laboratory recombinant virus lacking 3B1 and 3B2 also reduced viral RNA synthesis levels and infective particle formation *in vitro*, attenuates disease in pigs, but not drastically[7]. These studies indicated that 3B3 is more important than 3B1 and 3B2 to maintain viral RNA replication, but presence of all the three 3B copies helps in efficient RNA replication.

Materials and Methods

Amplification, Cloning, Expression of 3B3 Gene and Anti-Serum Production

The 3B3 gene was amplified using 3B (L): GCG CCC **GAA TTC**CAT ATG GGA CCC TAC GCC GGA CCG as a forward primer and 3B (R): GCG GAA TTC **AAG CTT** CTC AGT GAC AAT CAG GTT CTT as reverse primer. The amplified product of 213 bp was ligated into pET32A vector at *E coli* RI and *Hind*III sites for expression of 24 kDa fusion protein carrying 15 kDa N-terminal fusion and 9 kDa 3B3 in *E. coli* BL21 DE 3, pLys S cells. The expressed fusion protein was purified using Ni-NTA Aagarose column. Affinity purified 3B3 fusion protein was used for raising anti-serum in rabbits as per standard procedure. The animals were bled, serum separated from the whole blood, heat inactivated and stored. The reactivity was studied by enzyme linked immunosorbent assay (ELISA).

Deletion of 3B3 Gene from the Replicon

Gene sequence encoding 3B3 was deleted by PCR mutagenesis approach. Briefly, the amplified fragments corresponding to the region between 3A to 5' end of 3B3 and 3' end of 3B3 to middle of 3D. In order to introduce 3C cleavage site between 3B3 end and 3C, we have introduced the processing site in the primer along with the cloning sites. The sequences of the primers used for amplification are shown below.

1 3B3 link to 3C (L): GCG CCC GGG ACA CAA GGT GAGTGA TGA CGT GAA CTC CGA GCC CAC CAA ACC CGC GGA AGA GCA ACC ACA AGC TGA AAA AGC TAA GAA CCT GAT TGC CAC TGA GAG TGG TGC CCC ACC AAC CGA CTT 126 mer.
2 3D (R) Mlu: CGC GAC GCG TAG TCG GCA GCG CAG CGG CGG 30 mer.

Generation of BHK-21 Cell Clones Expressing FMDV 3B3

Generation of Transfer Vector for Inserting 3B3 into BHK 21 Cells

In order to achieve high 3B3 protein expression in baby hamster kidney (BHK) cell clones, we have selected cap-independent translation directed by FMDV internal ribosome entry site (IRES) approach. BHK FLP-In cell line and BHK cells expressing T7 polymerase under eEF1 promoter[(8)] were used for transfection and generation of stable 3B3 expressing cell clones. The peEF/ FRT plasmid was used as transfer vector to generate BHK 21 cell clones carrying either 3B3 gene or T7 RNA polymerase and 3B3 gene in bi-cistronic vector construct. The methodology is described below.

Cloning of 3B3 and T7 RNA Polymerase Genes in Transfer Vector

3B3 gene was amplified from the full length infectious cDNA construct using 3B (L): 5'GCG CCC GAA TTC CAT ATG GGA CCC TAC GCC GGA CCG 36mer as forward and 3B (R): 5'GCG GAA TTC AAG CTT CTC AGT GAC AAT CAG GTT CTT 36 mer as reverse primers similarly IRES of size 440 bp was amplified from full length cDNA of Asia 1 with IRES (L *Nhe*I/*Xba*1: 5' CGG AAT CCG CTA GCA GGT CTA GAG GGG TGA CAC CTT G 37 mer, and IRES (R-*Nde*I): 5' GCG AAT TCC ATA TGT AA A CAG TAG TGG TCA AAG G 34mer. primers. The PCR fragment corresponding to IRES was digested with *Nhe*I and *Nde*I and purified. The two fragments were ligated into the peEF/ FRT vector at *Nhe*1 and *Bam*H1 to get peEFIRES-3B3/FRT.

Cloning of T7 RNA Polymearase and 3B3 Genes into peEF/FRT Transfer Vector as Bicistronic Messenger RNA Producing Cassette

The T7 RNA polymerase gene was amplified and cloned into peEFIRES-3B3 at *Eco*RV and *Nhe*I sites. The transfer vector with T7 polymerase gene was designated as peEFT7-3B3-/FRT, which was used for transferring T7 and 3B3 into BHK 21 cells.

Generation of Stable BHK-21 Cell Lines Expressing Either 3B3 or T7 RNA Polymerase or 3B3 Under eEF1 Promoter

Generation of BHK cell clones expressing either 3B3 alone or 3B3 and T7 RNA polymerase were as per the protocol given by the manufacturer. Briefly, minimal lethal concentration hygromycin needed for the selection of recombinant BHK cells was determined for FLP-In 3B3 cell clone as done for Zeocin for the generation of FLP-In BHK cell clone.

BHK FLP-In 3B3 cell lines were transfected with 0.5, 1, 2 and 4 µg of peEF-IRES-3B3/FRT and peEFT7-3B3-/FRT plasmid separately along with pOG44 plasmid (carrying recombinase gene) in 1:9 ratio using transfectamine-2000 (Invitrogen). The hygromycin resistant clones (about 10) were further grown for two passages and the stable clones (6 no.) were grown and stored in liquid nitrogen before evaluation of integration and expression of 3B3 gene. The clones were further screened for the presence of 3B3 gene by PCR and 3B3 protein expression by western blot analysis. Of the three stable clones screened, one showed clear amplification of 3B3 and was used to rescue the virus in 3B3 deleted RNA transcripts of full-length infective cDNA clones. The presence of both T7 RNA polymerase gene and UTR-3B3 gene sequences were also screened by PCR. Further, functionality of the expressed T7 RNA polymerase in BHK cells was evaluated by transfecting the BHK-21 cell clones with pRSET GFP DNA. The positive clones showing the high GFP expression were designated as BHK 3B3 C2/2 cell line and BHK T7-3B3 2/1 cell line.

Infectivity Studies on the Transcripts of Full Length Constructs

Generation of Gene Deleted FMDV Chimeric 'O' cDNA Construction

Asia 1 replicon 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[9] Using this approach P1-2A of 'O' was introduced into the 3B3 gene deleted replicon at the created *Bam*HI site.

Development of Biosynthetic Viruses with Gene Deleted Constructs

BHK21 cell lines or cell lines expressing 3B3 (BHK-3B3 C2/2, BHK T7-3B3 C2/1) were 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 transfected with the plasmid, linearized at alternative restriction site (*Not*1) following poly (A) tract, using lipofectamine-2000 (Invitrogen) as per manufacturer's protocol. The infected cells were harvested by two freeze-thaw cycles, treated with 1% (v/v) chloroform at 4°C and the supernatant (500 µl) after centrifugation at 10,000 g for 10 min was used for subsequent passage into 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 into BHK21 cell lines. The cells were observed for cytopathic effect (cpe) for 24 hr post infection (PI). The FMDV in the cell supernatant was collected and stored at -70°C after chloroform treatment as described before.

iii) Characterization of the Biosynthetic Viruses

The biosynthetic 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

3B3 Gene Amplification and Expression

The amplified and cloned 3B3 DNA in pET32A prokaryotic expression vector was sequenced using T7 primer in ABI 377 automatic sequencer. A nucleotide sequence of 672 bases was obtained which includes 460 bases of vector sequence. The sequence was aligned with the published sequence. A homology of 94% was observed with the published sequence that corresponds to 3B3 gene (Fig. 1). The plasmid was designated as pET 3B3.

Figure 2 showed His-tagged protein could be eluted in the first two fractions (lane 1 & 2). No protein could be seen in the washout (lane 3). The size of the protein was 24 kDa that corresponded to the size of 3B3-protein of 9 kDa plus N-terminal tags of size 15 kDa. The protein was used for raising antiserum in rabbits.

Deletion of 3B3 Gene

The amplified 3C and part of 3D (-3B3 deleted gene) was used to replace the *Sma*I to *Mlu*I fragment of size 1.1 kb. One of the positive plasmid DNA was sequenced and the nucleotide sequence was subjected for BLAST search using NCBI data base. The sequence showed homology with the published FMDV Asia 1 sequence (Fig. 3) with 3 three regions. The 5' 1 - 459 nucleotides corresponds to 3A region (5401 to 5851 of full length Asia 1 IND isolate). While the region 483- 760 corresponds to 3C gene of FMDV Asia 1 isolates, region 6046 – 6345 indicating the deletion of 3B3 gene (5852 - 6045).

Generation of BHK-21 Cell Clones Expressing FMDV 3B3

peEF-T7-3B3 FRT transfer vector was used for stable transfection of FLP-In BHK 21 3C2 cell clone using lipofectamin 2000. About 6 clones were found to be resistant for hygromycin. One of them C2/1

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322 bits(174)        8e-85      200/213(94%)          0/213(0%)        Plus/Plus

Query  7    GGACCCTACGCCGGACCGCTCGAGCGTCAGAAACCTCCGAAAGTGAAAGCTAAGCTGCCA  66
            |||||||||||||||| || |||||||||||||| |||||||||||| |||||||||
Sbjct  460  GGACCCTACGCCGGACCACTTGAGCGTCAGAAACCTCTGAAAGTGAAAGCCAAGCTGCCA  519
Query  67   CAGCAGGAGGGACCCTACGCTGGCCCGATGGAGAGACAGAAACCACTGAAAGTGAAAGTG  126
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Sbjct  520  CAGCAGGAGGGGCCCTACGCCGGTCCGATGGAGAGACAGAAACCACTGAAAGTGAAAGCA  579
Query  127  AAAGCCCCGGTCGCGAAGGAAGGACCTTACGAGGGACCGGTGAAGAAGCCTGTCGCTTTG  186
            |||||||||||||  |||||||||||||||| ||||||||||||||||||||||||||
Sbjct  580  AAAGCCCCGGTCGTCAAGGAAGGACCTTACGAAGGACCGGTGAAGAAGCCTGTCGCTTTG  639
Query  187  AAAGTGAAAGCTAAGAACCTGATTGTCACTGAG  219
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Sbjct  640  AAAGTGAAAGCTAAGAACTTGATTGTCACTGAG  672
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Fig. 1 — Nucleotide sequence alignment of the 3B3 sequence with gene corresponding published sequence of Foot-and-mouth disease virus 'O' isolate O/HKN/1/75.

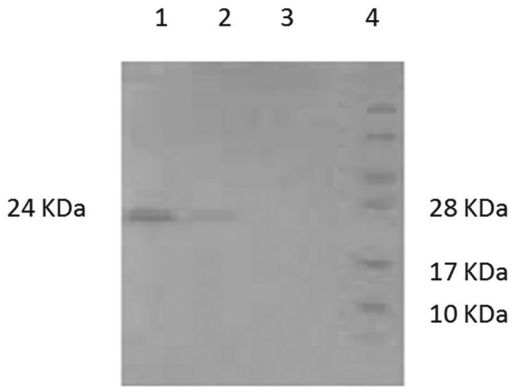

Fig. 2 — SDS-PAGE of affinity purified 3B3 fusion protein expressed in *E. coli.* Lane 1 & 2: Eluted Protein fractions, 3: Washout and 4: molecular weight markers.

(data not shown**)** was found to be positive for presence of both T7 and 3B3 by PCR. Interestingly, the cloned cells in the monolayer were found to be morphologically small and migrate rapidly to spread in the plate. The cells were resistant for hygromycin at 100 µg/ml concentration. Expression of T7 RNA polymerase was confirmed by studying the production of green fluorescent protein in pRSET GFP transfected cells. Expression of GFP was observed in transfected cells indicating the cloned cells were expressing T7 RNA polymerase and may be 3B3 (Fig. 4)

Infectivity Studies on the Transcripts of Full Length Constructs

Polyprotein coding region, P1-2A of FMDV 'O' was released from pBO-P1-2A with *Bam*HI and cloned into 3B3 gene-deleted full length Asia 1 63/72 replicon as described under methods. PCR positive colonies having P1-2A insert in 3B3 deleted full length construct was screened and the positive plasmid construct was designated as pBFLOΔ3B3. The pBFLOΔ3B3 were sequenced using reverse primer corresponding to 3A and presence of P1-2A of 'O' was confirmed. RNA transcript was produced from *Not*1 linearized construct using high yield RNA transcription kit and transfected into BHK cell monolayers at 30th passage. The cells were observed for cytopathic effect (cpe) 24 hr post transfection. As seen in the Figure 5, cpe (~60%) was observed in the case of full length transcripts from chimeric 'O' (FLO) (A). The monolayer in the case of FLO (-3B3)-transcript transfected (B) cells did not show any cpe indicating the RNA was not infective due the absence of 3B protein which is needed for replication.

The infectivity of the transcript from pBFLOΔ3B3 was tested in BHK cells expressing 3B3 under T7 polymerase (BHK T7-3B3-2/1). As seen in the Figure 6, the 3B3 expressing cloned cells (C2/2) showed foci with rounding of cells at 24 hr post transcription indicating the 3B3 expressed in 2/2 cells could rescue the virus replication externally giving raise to DISC virus in these cells.

Further, the efficiency of virus rescue after DNA transfection was studied in genetically modified recombinant BHK cells expressing T7 RNA polymerase and cells coinfected with recombinant vaccinia virus expressing T7 RNA polymerase (VT7). In the case of vaccinia system the recombinant vaccinia virus was allowed to infect BHK cells prior to transfection with the *Not*1 linearized DNAs (2 µg). The total RNA was extracted after second passage and 1 µg of RNA was used for transfection. The efficiency of virus rescue in the case of cells (clone 2/1) expressing T7 RNA polymerase was studied by transfecting 2 µg of *Not*1 linearized DNAs. Virus passages were studied for cpe. As seen in the Figure 7 more than 80% cpe was observed in the case of -3B3 at 1st passage (A, B). However the efficiency of virus rescue was high with vaccinia system (B) as compared to T7 RNA

Fig. 3 — Alignment of the nucleotide sequence of the PCR amplified gene construct carrying 3B3 deletion with the published full length genome sequence of type Asia 1 isolate IND 81-86.

polymerase expressing cell system (A). This may be due to the higher level of RNA polymerase production in the case of recombinant vaccinia virus system. Higher replication efficiency of FLO (-3B3) in 2/2 cells is interesting and this is due to the availability of 3B3 protein at optimum level helping the production of DISC virus.

The viruses need to be evaluated for immunogenicity and attenuation in cattle in safety test.

Discussion

It is a known fact that live attenuated vaccines elicit robust and long lasting immune response as compared to inactivated ones, as the replicating organism induces all arms of immune system resulting in better humoral and cellular responses against the pathogen. However, attenuation by natural selection is not a fool proof method as the point mutations that occur during passage may not be stable. This is more so in the case of viral pathogens like FMDV. With the present understanding on specific virulence factor(s) of a pathogenic virus and the availability of recombinant DNA technology has facilitated the creation of specific gene deleted pathogens for use as live vaccines. The approach of

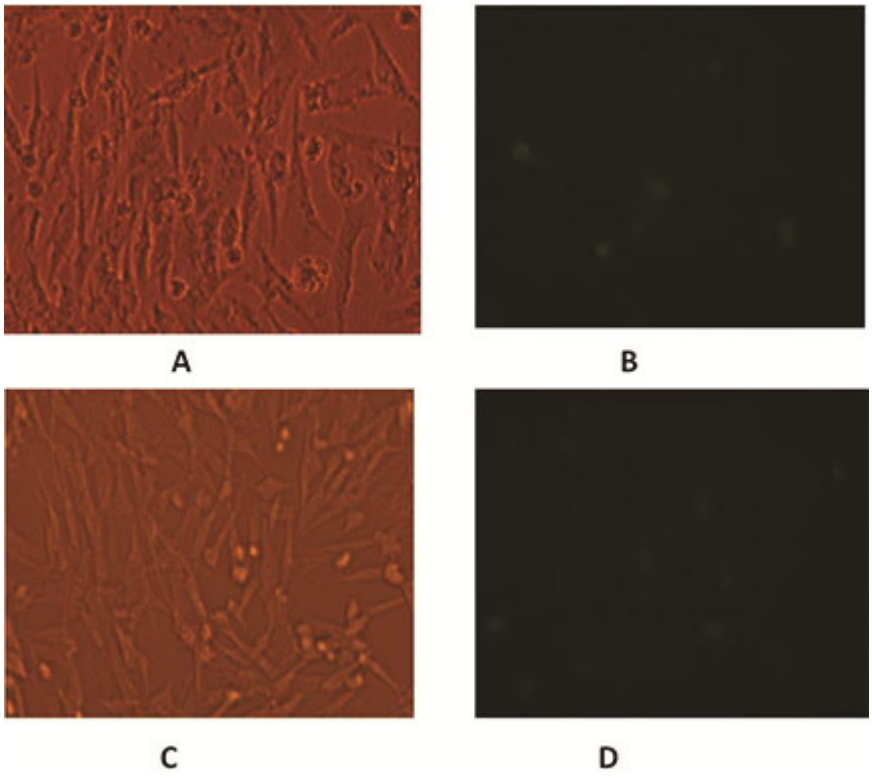

Fig. 4 — Expression of GFP in BHKT7-3B3-2/1 cells transfected with pRSET GFP. (a) pRSET DNA transfected BHK T7-3B3 2/1 cells visualized under white light at 10X magnification. (b) Visualized under fluorescent light under fluorescent microscope. C) and D) positive control

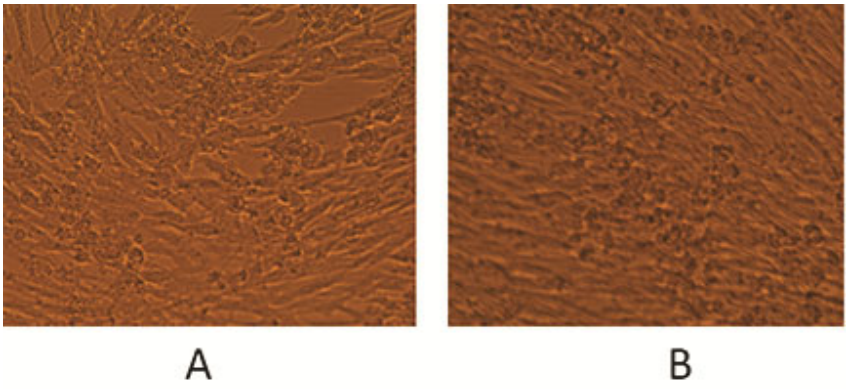

Fig. 5 — Infectivity of the transcripts of pBFLO (A) and pBFLOΔ3B3 (B) to wild BHK21 cells.

creating and testing defined gene deletions ultimately aids in reducing the pathogenicity/virulence of the organism without affecting the immunogenicity. Such gene-deleted organisms can be used as vaccines as they retain the immunogenic features of the wild-type organism but cannot cause disease. However, to be effective as viable vaccine (s), these organisms should be genetically stable, easy to grow and easy to administer. So far, genes involved either in determining virulence or regulating key metabolic pathways of the organism (s) have been targeted for such deletions. The recent advances in vaccinology has shown an approach of producing crippled virus that can replicate in the genetically manipulated host indefinitely and only once in vaccinated animal, thus inducing robust immune response against the virus without causing any disease. This is the basis of DISC virus approach. Here we have targeted 3B3 gene, which encodes 3 copies of virus-genome linked protein (VPg x 3) of 9 kDa, which after getting

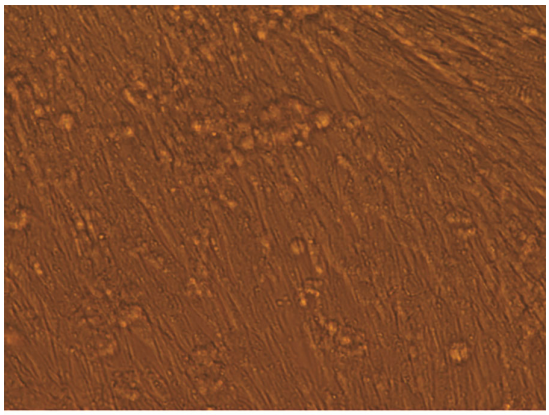

Fig. 6 — Infectivity of DNA transcript from pBFLOΔ3B3 to BHK cells 24 hr post tranfection.

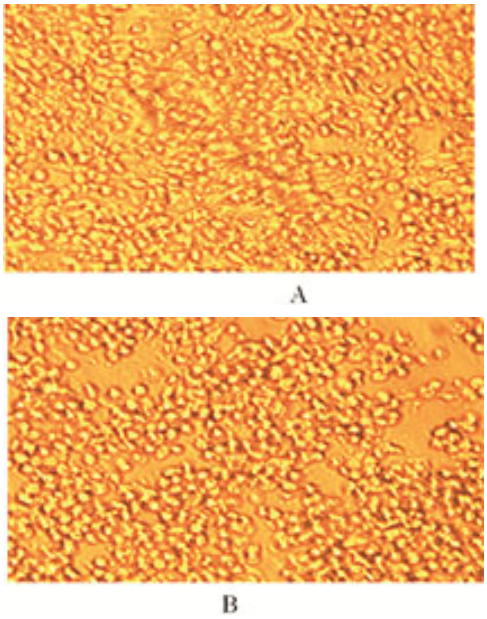

Fig. 7 — Infectivity of viral RNAs from rescued virus with 3B3 in recombinant BHK cells from *in vitro* transcribed DNA construct (A) and (B) with DNA coinfected with VT7.

processed by 3C, the virus specific protease, 3 kDa monomer acts as a primer which is essential for genome replication. We have successfully deleted 3B3 without changing the frame. However, we have left sequence encoding 8 amino acids in the junction at 3' end to facilitate cleavage of 3B3 and 3C junction. We have confirmed the deletions by sequence analysis and have successfully inserted 3B3 into BHK cell system to produce a clone stably expressing 3B3 and were used to rescue the virus. We have initially made deletions in the replicon developed by us and later inserted gene encoding P1-2A of 'O'. This methodology as described earlier facilitates the development of attenuated viruses of desired serotypes. We have tested the constructs for virus rescue and produced the viruses. The attenuated viruses were evaluated for infectivity using GFP gene in place of P1-2A. Initially a cassette carrying GFP gene followed by 2A and partial 2B so as to insert the GFP-2A-2B gene at single *Bam*HI site in the replicon. This was successfully done and confirmed by sequence analysis. The transcripts from the constructs were tested for infectivity in BHK cells expressing 3B3 gene. This has become necessary, since replication of the genome in 3B3 deleted transcript needs 3B3 and 3C. The 3C protease is expressed by the replicon while 3B3 is supplied by the 2/2 clone that expresses 3B3. As observed the GFP expression was seen with all the constructs indicating the intactness of the replicon for genome replication and expression of all virus specific proteins needed for replication. However, the interesting observation is that the 3B3 deleted construct showed the highest number of fluorescent spots, even though the amount of RNA used for transfection was the same. This may be because of high expression of 3B3 in the cells and the expressed protein helped in better replication. However, this should be the case with other constructs and should support better replication in the case of wild virus as compared to the gene deleted virus. Therefore, this could be inferred as RNAi effect of the 3B3 transcript produced in 3B3 expressing cells on replicons carrying 3B3 gene, thereby reducing the replication efficiency of intact genome. This observation may be used to develop therapeutic vaccines with 3B3 transcripts. The DISC virus produced using 3B3 as a rescue gene has a tremendous scope for attenuated vaccine approach and control of the disease.

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