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INHIBITION OF REPLICATION BY TARGETING SHRNAS AGAINST VP1 AND VP6 GENES OF BLUETONGUE VIRUS

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ABSTRACT

Background: Bluetongue virus (BTV), a member of Orbivirus genus within the *Reoviridae* family causes a haemorrhagic disease mainly in sheep and is transmitted between its mammalian hosts by certain *Culicoides spp*. Until now 26 serotypes have been identified worldwide. Many vaccine strategies developed to control the disease are not yet completely successful because of their inability to offer cross protection. RNA interference (RNAi) is the process by which double-stranded RNA directs sequence-specific degradation of homologous mRNA.RNA interference has been used to induce gene silencing in a large number of viruses but not attempted to BTV. **Results:** The shRNA producing cassettes targeting genes expressing VP1and VP6 proteins of BTV were obtained on successful cloning in psiRNA vector. Initial studies were carried out by transfecting the BHK21cells followed by infecting with four BTV serotypes [BTV1, 10, 16 and 23; (8h post infection)] at 100 $TCID_{50}$ and 1000 $TCID_{50}$ virus and harvested at 36h p.i. Upon titration, inhibition of replication was observed in cells transfected with shRNA producing cassettes of VP1 and VP6. The results of this study indicated that knocking down of VP1 gene (polymerase) and VP6 gene (helicase) of BTV is effective on inhibition of replication of BTV serotypes and the shRNA is effective upto 36h p.i. **Conclusion:** This study demonstrates that vector based shRNA methodology can effectively inhibit replication of bluetongue virus in BHK21 cells and can be used to control spread of BTV when used *in vivo*.

KEY WORDS: RNAi, shRNA, BTV, $TCID_{50}$

Background

Bluetongue (BT) has become one of the important sheep diseases of the Indian sub-continent. The first outbreak of BT in India was recorded in 1964 among sheep and goats in Maharashtra State, on the basis of clinical signs and detection of B TV antibodies in the sera o f animals which had recovered[1]. The disease has since become established in India, a geographically vast and climatically diverse country. *Culicoides* insects are the vectors of BTV. BTV is a complex non-enveloped virus with seven structural proteins and an RNA genome consisting of 10 double-stranded (ds) RNA segments of different sizes, each coding for individual proteins. The outer capsid is composed of two major structural proteins (VP2 and VP5), is involved in cell attachment and virus penetration during the initial stages of infection. The inner capsid is composed of two major proteins VP7 and VP3, and the three minor proteins VP1, VP4 and VP6 in addition to the dsRNA genome. In addition to the seven structural proteins, three non-structural (NS) proteins, NS1, NS2, NS3 (and a related NS3A) are synthesised in BTV-infected cells. Of these, NS3/NS3A is involved in the egress of the progeny virus. The two remaining non-structural proteins, NS1 and NS2, are produced at high levels in the cytoplasm and are believed to be involved in virus replication, assembly and morphogenesis[2].

For the control of the disease both inactivated and live attenuated vaccines are being used in various countries. Since viruses with RNA genomes such as BT virus (BTV) have high frequency of mutations, use of live virus vaccines is limited and discouraged because of possible genetic recombination in the vector or host with possibility of a new serotype or more virulent virus. With limited cross protection between these multiple serotypes, the usefulness of inactivated vaccines is also limited. Ribonucleic acid (RNA) interference (RNAi) is a naturally occurring intracellular mechanism, which causes sequence specific post transcriptional gene silencing. The reaction is triggered by the introduction of double-stranded (ds) RNA into the cytoplasm of the cell, and results in the specific targeted destruction of mRNA and a subsequent reduction in protein production[3]. RNA interference mechanism, can be used as a therapeutic

effective for all the 26 serotypes A preliminary study was undertaken to assess the usefulness of RNAi mechanism using shRNA in inhibiting the BTV replication in *in vitro* by targeting shRNA against VP1 and VP6 genes of BTV.

MATERIALS AND METHODS

Viruses and cells

Bluetongue virus serotypes used in this study were BTV1, 10, 16 and 23. They were maintained in BHK21 cell line. The cells were cultured in Eagle's minimum essential medium (Glasgow modifications) medium (GMEM; Gibco-BRL) supplemented with 5% heat inactivated foetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). Cultures were incubated at 37°C with 5% CO2.

Construction of shRNA producing cassettes

Oligos for small interfering RNAs for two genes of bluetongue virus (VP1, and VP6) were designed using the online software "siRNA Wizard" (*Invivogen*). Nucleotide sequences of each gene were selected from GenBank (gi 438501 for Bluetongue Virus VP1 gene, gi 194305008 for Blue tongue Virus VP6 gene). Selected siRNA oligos are as follows

For VP1 gene: VP1shRNA1: 5'GAGGTGTACGTGAACTCAATT3'; VP1shRNA2: 5'GCTGATTGCTTTCGTAACTCT3'

For VP6gene: VP6shRNA1: 5'GCGTTCATCCGAGGAGTTAAA 3'; VP6shRNA2: 5'GGAGGAAGATGGGTAGTTCTA 3'

Two shRNA oligos for each gene were designed by same software for cloning at Bbs1/Bbs1of psiRNA vector. These oligos were procured from Bioserve Ltd. Complementary oligonucleotides of each of hairpin inserts were annealed to form double stranded palindromic DNA. The vector used for the study was psiRNA-h7SK zeo G1 (Invivogen). The cloning strategy is shown in figure 1. The vector (psiRNA) DNA was digested with Bbs1 restriction enzyme by keeping at 37^0 C overnight in suitable buffer and incubation the enzyme was inactivated by heating at 65^0C for 20 min. The restricted vector DNA was electrophoresed through preparatory 0.7% low melting point agarose gel along and the larger fragment (2529bp) was cut out from the gel and eluted through Gene Jet™ Gel extraction kit (Fermentas) as per the manufacture's protocol. The annealed shRNA oligos having the Bbs1 overhangs were ligated into the Bbs1 digested psiRNA vector by incubating at 16^0 C overnight and the enzyme was inactivated by heating at 65^0 C for 10min. Transformation was carried out for four ligated vector constructs in *E. coli* (DH5α) and white recombinant colonies were selected on LB agar plate (X-gal, IPTG and Zeocin) by incubating at 37^0 C overnight. The recombinant were screened by colony PCR using vector specific primers (OL599, OL408) and confirmed by PCR, Pst1 digestion and sequencing (Chromous Biotecch Ltd.). Once the clones were confirmed for the presence of shRNA sequences in psiRNA vector of VP1 and VP6 constructs, the DNA was further isolated using Endotoxin Free DNA isolation Kit (Endo Free Plasmid Maxi kit, Qiagen).

Transfection of the shRNA expression cassettes into BHK-21 cells

BHK-21 cells were seeded in 24 well plates and cultured at 37°C and 5% CO2 overnight. When the cells showed 70-80% confluence, 0.8µg DNA (VP1shRNA1,VP1shRNA2, VP6shRNA1 and VP6shRNA2) was diluted in 50µl of Opti-MEM1 Reduced Serum Medium without serum (Invitrogen) and 2µl of Lipofectamine 2000 was diluted in 50µl of Opti-MEM1 Reduced Serum Medium and incubated for 5 min at room temperature and both solutions were combined and incubated for 20 min at room temperature for the formation of Lipofectamine-DNA complex. To each well containing cells 100µl of Lipofectamine 2000 - DNA complex were added slowly drop by drop after washing the cells with antibiotic free GMEM and mixed by rocking the plate back and forth. Non-transfected BHK-21 cells were also used as a control. A set of wells were transfected with the purified pcDNA GFP plasmid to serve as a measure for transfection efficiency. The cells were incubated at 37^0 C in CO_2 incubator. Transfection complex was removed 3-4h post transfection and replaced with antibiotic free growth medium with 2% FCS and again incubated at 37^0 C in CO_2 incubator.

Infection of BTV serotypes

Following transfection with shRNA producing cassettes, the monolayers were infected with various BTV serotypes to study the inhibition on virus replication. Infection was carried out separately with four serotypes BTV-1, BTV-10, BTV-16, and BTV-23 to the transfected cells at two virus concentrations as per standard procedure. Briefly to the transfected wells, 100µl of 100 $TCID_{50}$ and 1000 $TCID_{50}$ virus particles of BTV serotypes were added separately 8 h of post transfection, after removing the growth medium. The plates were kept for virus adsorption in CO_2 incubator at 37^0C for 1h with intermittent shaking. After one hour of adsorption, the excess virus was washed with serum free medium and incubated in GM with 2% FCS. The plates were incubated in CO_2 incubator at 37^0C and harvested at 36h of post infection by three cycles of freezing and thawing.

Analysis of pcDNAGFP expression in BHK-21 cells

After an additional 24 h of incubation, cells were observed for the expression of green fluorescent protein in the transfected cells was monitoring fluorescent microscope.

Virus titration

To determine transfection efficiency, we monitored GFP fluorescence intensity of transfected cells using fluorescent microscope analysis. Effect of shRNA producing cassettes on replication was studied by carrying out titration of the virus at 36h p.i. Culture

supernatants were collected for virus titration. Virus infectivity was determined by serial dilutions of the samples in 96-well plates and the virus titer was calculated as a $TCID_{50}$ by the Reed-Muench method[4]. Observations were taken for CPE up to 6th day post infection. $TCID_{50}$ for each transfected samples were calculated and compared with $TCID_{50}$ of non transfected virus control.

RESULTS

Screening of recombinant shRNA producing cassettes

A number of white colonies were seen on the X-gal, which may represent vector with shRNA insert. There were no blue colonies indicated that the recombination efficiency was about 100% or the vector linearization was 100%. Since there is a sequence difference in the overhangs of the Bbs1 sites there will be no self ligation and hence no blue colonies are expected to appear. Colony PCR was carried out for a number of selected white colonies with vector specific primers OL408, OL559 which corresponds to the sequences within the vector. The clones which produced a specific band of around 270bp (figure2) were considered as positive clones. Plasmid DNAs of four positive clones and the vector were digested with Pst1 restriction enzyme (figure3). Release of 340bp fragment indicates that the clones are positive (lane 2-7) for the presence of inserted shRNA cassette. However on digestion of the vector alone, a fragment of size 625bp size could be seen in the gel (lane 8). DNAs from positive clones were sequenced with forward and reverse primer and confirmed for insert.

Transient cellular transfection in BHK-21 cells with shRNA producing cassettes

BHK_{21} cell lines were transfected with shRNA producing cassettes of VP1shRNA1, VP1shRNA2, VP6shRNA1 and VP6shRNA2 using Lipofectamine 2000 in 24 well plates as per standard protocol. The transfectd cells were further infected with BTV serotypes 1, 10, 16 and 23 at 8h post transfection. The transfection efficiency of the positive vector constructs were assessed using pcDNA GFP vector. Presence of green florescence (figure 3) in the cells indicates that the transfection has occurred and the GFP expressed in the cells and the efficiency was observed as 80%. Hence the efficiency of transfection in case of shRNA constructs for the psiRNA clones may be considered as 80%. The cells were found to be healthy after transfection (figure 4) indicated that the DNA constructs were non toxic to the cell.

Interference of BTV replication by shRNA expression vector

Virus titration was carried out at 36h p.i. to study the effect of different shRNA producing cassettes on inhibition of virus replication as per standard procedure.

Effect on replication of BTV1 serotype

The BTV1 produced in transfected and infected (100 $TCID_{50}$) cells were titrated; $TCID_{50}$ was calculated and compared with the virus control value to know the level of inhibition on replication of BTV1. The titre of the virus was reduced to $< {}_{10}$Log 1 per 100µl of sample in cells which were transfected with VP1shRNA1, VP6shRNA1 and VP6shRNA2 but the cells transfected with VP1shRNA2 $_{10}$Log 3.44. However, the virus titre in absence of any shRNA was $_{10}$Log 4.4 per 100µl of sample. When the cells were infected with 1000 $TCID_{50}$ of BTV1, inhibition on replication was observed in the case of cells transfected with VP1shRNA1 where the titres were to less than $_{10}$Log1 per100µl. But transfection with VP1shRNA2 did not show appreciable reduction in the virus titre ($_{10}$Log 6) indicating that this shRNA may not be specific for BTV1. Interestingly there was a decrease in titre of BTV1 in the case of cells transfected with VP6shRNAs ($_{10}$Log 1 for VP6shRNA1, $_{10}$Log 1.36 for VP6shRNA2), indicating that these shRNA constructs showed good inhibitory effect on virus replication.

Effect on replication of BTV10 serotype

The transfected cells were infected with 100 $TCID_{50}$ of BTV10, the titre value for VP6shRNA1 was less than $_{10}$Log1 per 100µl of sample while that with VP6shRNA2 the titre was given $_{10}$Log 1.6 per 100µl of sample, while the titre in the case of mock transformed cells was 2.5. The BTV10 virus titres in the case of VP1shRNA1 & 2 transfected cells were not effective as the titres were 3.4 and 3.28 respectively.

The virus control for BTV10 at an infection dose of 1000 $TCID_{50}$ showed a titre of $_{10}$Log 3.4. Only the cells transfected with VP1shRNA2 gave titre less than $_{10}$Log1. The titre of virus in cells transfected with VP6shRNA1 and VP6shRNA2 was $_{10}$Log 2.6 and 1.74 respectively and a reduction in titre was observed in cells transfected with VP1shRNA1 also ($_{10}$Log 2.5) indicating these constructs has inhibitory effect on BTV 10 replication similar to BTV 1.

Effect on replication of BTV16 serotype

When the cells were transfected and infected with 100 $TCID_{50}$ of BTV16, the cells which were transfected with VP6shRNA1 showed a virus titre of less than $_{10}$Log1. Similarly the titre was $_{10}$Log 1.31 and 1.5 observed in the case of cells transfected with VP6shRNA2 and VP1shRNA2 respectively. The cells transfected with VP1shRNA1 has shown a titre of $_{10}$Log 3.8. The $TCID_{50}$ of the mock transfected cells was $_{10}$Log 3.4 per 100µl of sample.

There was a very good reduction in the titre of BTV16 when infected at 1000$TCID_{50}$ to the cells transfected with VP1shRNA1 and VP6shRNA2 ($< {}_{10}$Log 1). The titre in the case of mock transfected cells was $_{10}$Log3.5 per 100µl of sample and was same for VP1shRNA1 indicated that there was no inhibition on replication. The cells transfected with VP6shRNA1 showed reduction in titre of $_{10}$Log 3.31 indicates there was an inhibition in virus replication.

Effect on replication of BTV23 serotype

Cells transfected with VP1shRNA1 & 2 when infected with BTV23 at 100 $TCID_{50}$, the titres were $_{10}$Log 1.6 and $_{10}$Log1.7 respectively. The titre value of virus for cells transfected with VP6shRNA1 &2 also showed a reduction ($_{10}$Log 1.5 and $_{10}$Log 1) as the titre for virus control was found to be $_{10}$Log 2.8 per 100µl of sample.

There was a very good reduction of titres up to less than 10 Log 1 observed for cells transfected with VP6shRNA1 and VP6shRNA2 when the cells were infected with 1000$TCID_{50}$ of BTV23. The cells transfected with VP1shRNA1 ($_{10}$Log 1.8) and with VP1shRNA2 ($_{10}$Log1.7) showed reduced in titre than virus control ($_{10}$Log 3.5). Titres for different serotypes of BTV at 100$TCID_{50}$ and 1000$TCID_{50}$ doses were given in table 1 and overall analysis in table 2.

Table 1: Virus titre in cells transfected with different shRNAs and infected with different BTV serotypes at 100 and1000 $TCID_{50}$ (36h p.i.)

Sl. No.	Sample	Serotypes of BTV							
		BTV1		BTV10		BTV16		BTV23	
		100 $TCID_{50}$	1000 $TCID_{50}$	100 $TCID_{50}$	1000 $TCID_{50}$	100 $TCID_{50}$	1000 $TCID_{50}$	100 $TCID_{50}$	1000 $TCID_{50}$
1	VP1shRNA1	<1	<1	3.4	2.5	3.8	3.5	1.6	1.8
2	VP1shRNA2	3.44	6	3.28	<1	1.5	<1	1.7	1.7
3	VP6shRNA1	<1	1	<1	2.6	<1	3.31	1.5	<1
4	VP6shRNA2	1	1.36	1.6	1.74	1.31	<1	1	<1
5	Virus control	**4.4**	**3.6**	**2.5**	**3.4**	**3.4**	**3.5**	**2.8**	**3.5**

(Virus titre expressed in $_{10}$Log values)

Table 2: Effect of different shRNAs in inhibiting replication of different BTV serotypes at 100 and1000 $TCID_{50}$ (36h p.i.)

Sl. No.	Sample	Serotypes of BTV							
		BTV1		BTV10		BTV16		BTV23	
		100 $TCID_{50}$	1000 $TCID_{50}$	100 $TCID_{50}$	1000 $TCID_{50}$	100 $TCID_{50}$	1000 $TCID_{50}$	100 $TCID_{50}$	1000 $TCID_{50}$
1	VP1shRNA1	+	+	-	+	-	-	+	+
2	VP1shRNA2	+	-	-	+	+	+	+	+
3	VP3shRNA1	-	-	-	-	-	-	-	-
4	VP3shRNA2	-	-	-	-	-	-	+	+
5	VP6shRNA1	+	+	+	+	+	+	+	+
6	VP6shRNA2	+	+	+	+	+	+	+	+

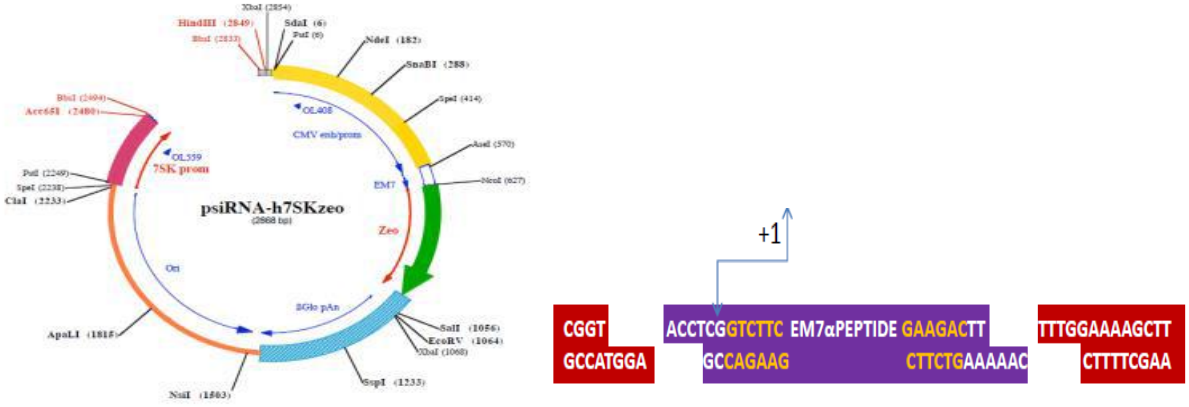
Note : + for inhibition
- for no inhibition

Step 1.Selection of siRNAsequence using siRNAWizard software
5’ GAGGTGTACGTGAACTCAATT 3’ siRNAsequence for VP1shRNA1

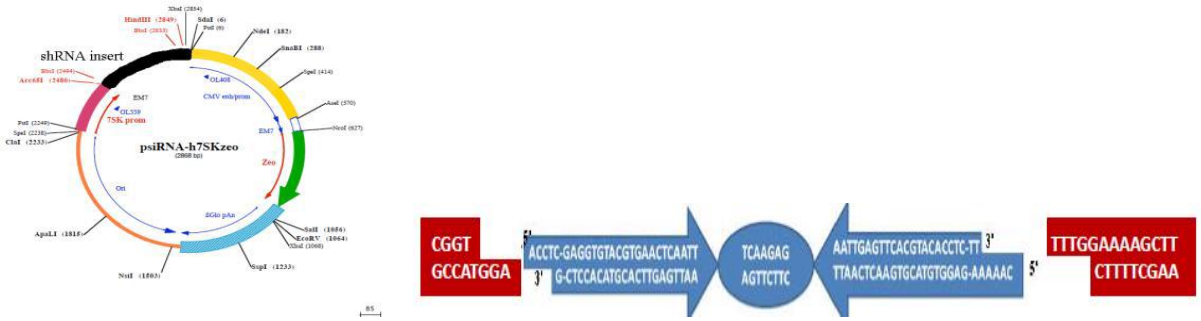
Step2. Synthesis of complementary oligonucleotides
Oligo15' ACCTCGAGGTGTACGTGAACTCAATTTCAAGAGAATTGAGTTCACGTACACCTCTT 3‘Oligo25' CAAAAAGAGGTGTACGTGAACTCAATTCTCTTGAAATTGAGTTCACGTACACCTCG 3’

Step3. Annealing of complementary oligonucleotides

Step4. Digestion of psiRNAplasmid with Bbs1



Step5. Ligation and transformation to *E.coli*DH5α



Recombinant vector plasmid

Fig. 1. Cloning strategy

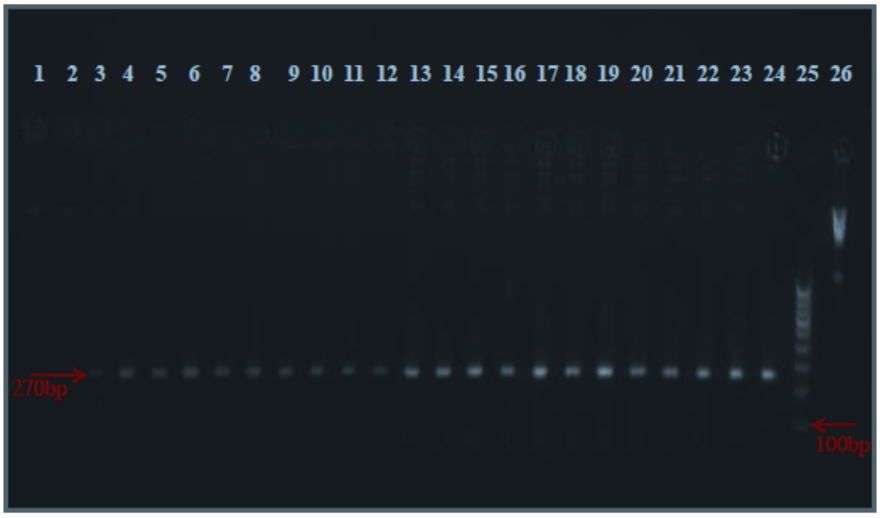


Figure 2. Agarosegel electrophoresis confirming the recombinant colonies by colony PCR showing 270bp band (lane1-24)

Lane 1-4: VP1shRNA1 recombinant colonies,
Lane5-8: VP1shRNA2 recombinant colonies,
Lane9-12: VP3shRNA1 recombinant colonies,
Lane 13-16: VP3shRNA2 recombinant colonies,
Lane 17-20: VP6shRNA1 recombinant colonies,
Lane21-24: VP6shRNA2 recombinant colonies,
Lane25: 100bp DNA ladder,
Lane26: 1kb DNA ladder

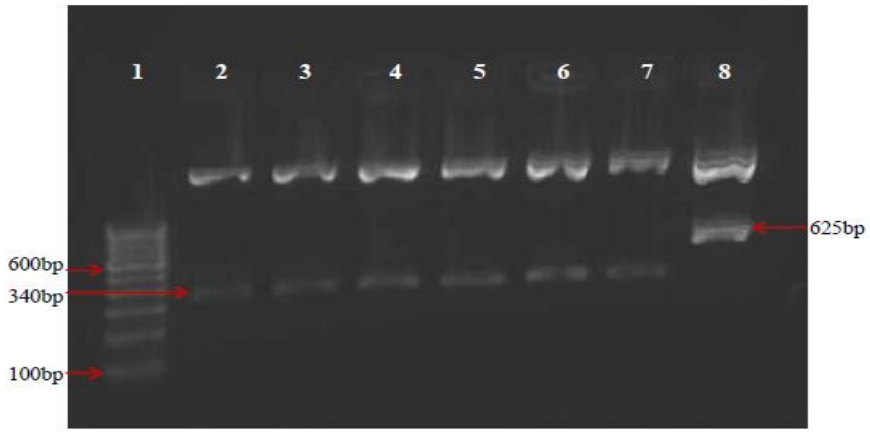


Figure 3: Agarosegel electrophoresis showing the positive clones releasing 340bp fragment by Pst1 digestion

Lane 1 -100bp DNA ladder,
Lane2 -VP1shRNA1,
Lane 3 -VP1shRNA2
Lane 4 -VP3shRNA1,
Lane 5 -VP3shRNA2,
Lane 6 -VP6shRNA1
Lane 7 -VP6shRNA2,
Lane 8 –psiRNAvector releasing 625 bpfragment on Pst1 digestion

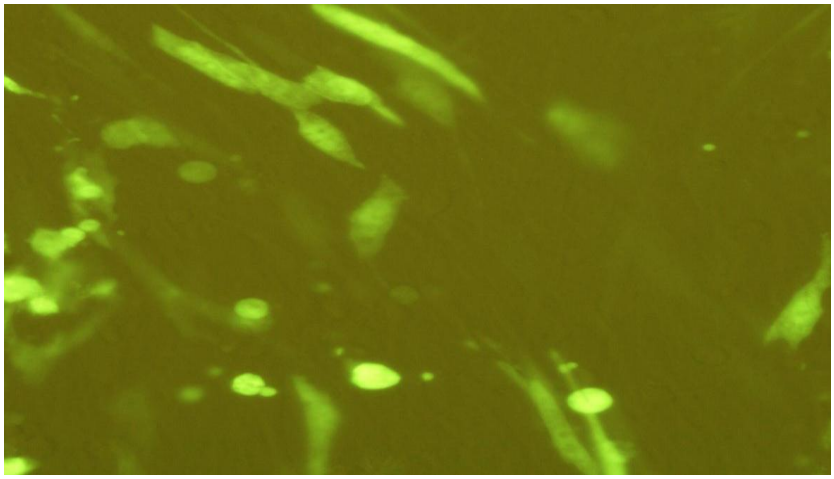


Figure4: Fluorescence given by BHK21 cells transfectedwith pcDNAGFP indicating the efficiency of transfection

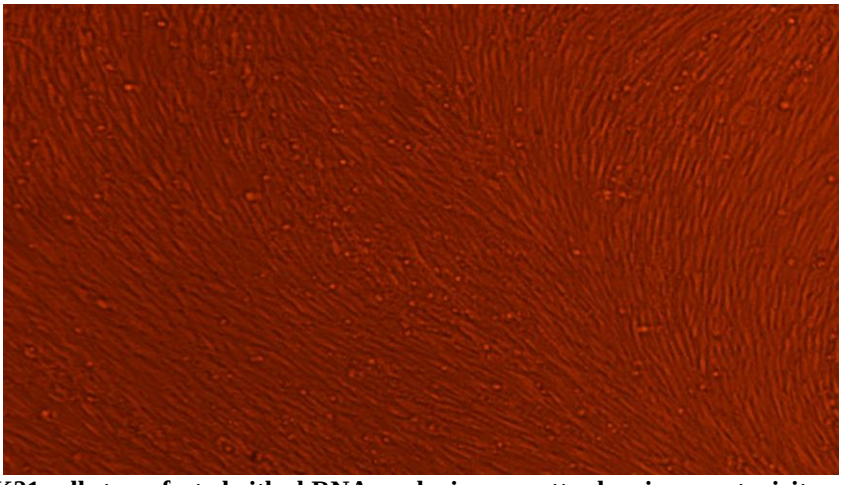


Figure 5: BHK21 cells transfectedwith shRNAproducing cassette showing non toxicity on transfection

DISCUSSION

RNAi is a process of sequence-specific, post-transcriptional gene silencing that is initiated by double stranded RNA. Introduction of siRNA results in degradation of siRNA specific transcripts thus reducing the expression of their protein product. In plants, it is a natural antiviral defense mechanism[5]. In mammalian cells, however, dsRNAs longer than 30 nt activate an antiviral defense leading to the nonspecific degradation of RNA transcripts and a general shutdown of host-cell protein translation[6]. The successful use of siRNA in animal cells encouraged the development of siRNA expression vector[7] and numerous studies have demonstrated that DNA-based siRNA is a promising approach for antiviral therapy in mammals. Studies by Joyappa *et al*.[8] suggested that shRNA could be a viable therapeutic approach to control severity of FMD infection and spread.

VP1 an RNA dependent RNA polymerase is one of the most important proteins required for viral replication (Boyce *et al.*, 2004) and VP6 binds to ssRNA and dsRNA with helicase function (Stauber *et al.*, 1997). Matsuo and Roy (2009) showed that VP6 is active early in replication, consistent with a role as part of the transcriptase or packaging complex, and that its action can be provided in *trans* by a newly developed complementary cell line. The present study was taken up for targeting these three genes as inhibition of these genes inside the cell affects the virus replication, maturation and release.

Different siRNA sequences display widely different efficacies with regard to suppression of gene expression, requiring screening of multiple sequences[12]. In this research, we have selected four target sequences for RNA interference was designed using the "siRNA Wizard" software (Invivogen), because the vector was developed by Invivogen.

CONCLUSION

This work was an initial step towards the control of BTV using shRNA mediated RNAi. Knocking down of VP1 and VP6 genes has given good results for inhibition of virus replication. The work shows that there is scope for screening of more shRNAs and combinations of shRNA for getting a better inhibition. In conclusion, this study demonstrates that vector-based shRNA methodology can effectively inhibit BTV replication on BHK21 cells. Further study will be required to determine whether such treatment protect against BTV infection *in vivo*.

Abbreviations

BTV: Bluetongue virus; RNAi: RNA-mediated interference; siRNA: Short interference RNA; shRNA: Short hairpin RNA; GFP: Green fluorescent protein; FCS: Fetal calf serum; p.i.: Post-infection, p.t.: post transfection; BHK-21: Baby Hamster Kidney cells.

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Competing interests

The authors declare that they have no competing interests.

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