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COMBINATIONS OF SHRNA INHIBITS BTV REPLICATION *IN VITRO* IN BHK21CELLS

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ABSTRACT

Bluetongue is an economically important disease for the farming community however vaccines effective for all 26 serotypes are unavailable. Hairpin expression vectors have been used to induce gene silencing in a large number of studies on viruses. However, none of these studies has been attempted to study BTV. In this study we show the suppression of bluetongue virus (BTV) replication by targeting combinations of short hairpin (sh) RNAs against VP1, VP3 and VP6 genes. Two siRNAs were selected for conserved regions of VP1, VP3 and VP6 gene and corresponding shRNA producing cassettes were obtained on successful cloning in psiRNA vector. Endotoxin free plasmid DNAs of positive clones were used for transfection in BHK_{21} cell lines. Transfection was followed by infection with 100TCID50 of BTV1 serotype 8 h Post transfection (Pt). All of them were found to be effective up on titration at 48 hour post infection (Pi). The threshold cycle (Ct) values on quantitative Real Time PCR of the above sample indicated that inhibition was achieved by all the combinations of shRNAs. The results of the present study revealed that knocking down of VP1, VP3 and VP6 genes will be effective for the control of BTV multiplication by transfection with combinations of shRNA producing cassettes in vitro in BHK21 cells. This study demonstrates that combinations of shRNA can effectively inhibit BTV replication *in vitro* in BHK21 cells.

Key words: RNAi, shRNA, BTV, $TCID_{50}$, Ct value

[I] INTRODUCTION

Bluetongue virus (BTV) is a member of Orbivirus genus within the *Reoviridae* family has been in the forefront of molecular studies for the last three decades and now represents one of the best understood viruses at the molecular and structural level. 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. Considerable advances have been made in recent years in understanding the replicase complexes of BTV [1]. The RNA replicase activity of purified VP1 protein has been demonstrated and observed that the replicase activity consisted of *de novo* initiation of synthesis, followed by elongation of the minus strand and exhibited little sequence specificity for BTV plus-strand template, suggesting that the choice of viral over non viral RNA template comes from its association with other proteins within the viral core [2]. For virus assembly and replication inside the cell function of VP3 is crucial. The findings of Kar *et al.* [3] about VP3 localization and its fate within the host cell with assembly, capability of a VP3 molecule with a large amino-terminal extension explores the possibility of application as a delivery system. The other functional protein VP6 of bluetongue virus possesses a number of activities, including nucleoside triphosphatase, RNA binding, and helicase activity [4] and it exits as multiple oligomeric forms.

Cross protection between multiple serotypes limit the usefulness of vaccines used at present. Hence it is necessary to develop control measure which should be effective on all the 26 serotypes. One of such novel approaches would be intervention in the viral replication using RNA interference mechanism, which can be used as a therapeutic. 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 [5].

RNA mediated interference (RNAi) is a conserved gene-silencing mechanism, where by the double stranded RNA matching is used as a signal to trigger the sequence-specific degradation of homologous mRNA [6]. RNAi represents a new antiviral method and is being increasingly used to inhibit the replication of viral pathogens [7]. Hairpin expression vectors have been used to induce gene silencing in a large number of studies on viruses [8-12].

This is the first report of use of combinations of shRNA for inhibiting the replication of BTV1 by knocking down the genes involved in replication of the virus. The work shows that there is scope for screening of more shRNAs combinations for getting a better inhibition and further in *in vivo* experiments in sheep.

[II] MATERIALS AND METHODS

2.1. Viruses and cells

Bluetongue virus serotype used in this study was BTV1 and was 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.

2.2. Construction of shRNA producing cassettes

Oligos for small interfering RNAs for three genes of bluetongue virus (VP1, VP3 and VP6) were designed using the online software "siRNA Wizard" (*Invivogen*). Nucleotide sequences of each gene were selected from GeneBank (gi 438501 for Bluetongue Virus VP1 gene, gi 438515 for Bluetongue Virus VP3 gene, gi 194305008 for Blue tongue Virus VP6 gene). Selected siRNA oligos for are as follows

For VP1 gene:

VP1shRNA1:
5'GAGGTGTACGTGAACTCAATT3';
VP1shRNA2: 5'GCTGATTGCTTTCGTAACTCT3'
For VP3 gene:
VP3shRNA1:5 'GCATTCGCATAATTTCATCAA3',
VP3hRNA2: 5 'GCACTGTACGCGTTAAATTCT3'
For VP6gene:VP6shRNA1:

5'GCGTTCATCCGAGGAGTTAAA3';
VP6shRNA2: 5'GGAGGAAGATGGGTAGTTCTA3'
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 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, VP3 and VP6 constructs, the DNA was further isolated using Endotoxin Free DNA isolation Kit (Endo Free Plasmid Maxi kit, Qiagen).

2.3.Transfection of the shRNA expression cassettes into BHK-21 cells

Transfection was carried out with 12 combinations of VP1shRNA1+VP3shRNA1,
VP1shRNA1+VP3shRNA2,
VP1shRNA2+VP3shRNA1,
VP1shRNA2+VP3shRNA2,
VP1shRNA1+VP6shRNA1,
VP1shRNA1+VP6shRNA2,
VP1shRNA2+VP6shRNA1,
VP1shRNA2+VP6shRNA2,
VP3shRNA1+VP6shRNA1,
VP3shRNA1+VP6shRNA2,
VP3shRNA2+VP6shRNA1 and
VP3shRNA2+VP6shRNA2.

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 (4µg DNA each from individual shRNA producing cassettes) 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.

2.4. **Infection of BTV1 serotype**

Following transfection with shRNA producing cassettes, the monolayers were infected with BTV1 serotype to study the inhibition on virus replication. Transfected cells were infected BTV-1 at 100 $TCID_{50}$ virus concentration 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 48h of post infection by three cycles of freezing and thawing.

2.5. 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.

2.6. Virus titration

Effect of shRNA producing cassettes on replication was studied by carrying out titration of the virus at 48h p.i. Virus infectivity was determined by serial dilutions of the samples in 96-well plates and the virus titre was calculated as a $TCID_{50}$ by the Reed-Muench method [13]. Observations were taken for CPE up to 6^{th} day post infection. $TCID_{50}$ for each transfected samples were calculated and compared with $TCID_{50}$ of non transfected virus control.

2.7. Quantification of virus by qRT- PCR

Primers for qRT- PCR was designed for BTV-NS3 gene using Primer 3 software. The gene sequence used for primer designing was taken from NCBI database >gi|215276343|gb|FJ437561.1used for designing primer. Published primers sequences for Beta Actin gene [14] were selected and used in the studies. The primers were reported to have 100% homology with BHK Beta Actin gene.

NS3 Forward primer - 5'GGAACGGGTTGAAGAGTTGA 3'

NS3Reverse primer - 5'GCAACAGTAGGCATTGACGA 3'

Beta-actin- Forward primer -5' AGCCGAGAGGGAAATTGT 3'

Beta- actin- Reverse primer - 5' CCAGGGAGGAAGAGGATG 3'

RNAs of cells harvested (48h pi) from transfected and infected BHK21 cells using Trisoln reagent (GeNei). cDNA was synthesised for NS3 gene of BTV and for Beta Actin primers in 2 step reaction using MMuLV reverse transcriptase enzyme (Invitrogen) from all the RNA samples. Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas®) was used for Real Time PCR in Real Plex (Eppendorf®) in 96 well plates (Twin Tec Real Time PCR Plates 96). The reaction mix was prepared by adding Maxima® SYBR Green qPCR Master Mix (2X) 10 µl, primers (20pM, 0.2 µl), Template cDNA (1µl), nuclease-free water to make up 20 µl. The reaction cycle was an initial denaturation of 95^0 C for 5 min, cycling denaturation of 95^0 C for 15 sec, annealing at 48^0 and 50^0 C for 30 sec for NS3 and Beta Actin respectively and extension at 72^0 C for 30 sec. 40 cycles were done and succeeded by a melting curve of 65^0 C to 95^0 C with 0.5^0 C increases in every 5 seconds.

2.8. Data analysis of qRT PCR

The Data obtained from the software was analysed by comparative Ct method [15]. The amount of target normalized to an endogenous reference and relative to a control sample was given by the formula,

$$\frac{X_{test}}{X_{control}} = 2^{\Delta\Delta C_T} = 2^{\Delta C_{T,control} - \Delta C_{T,test}}$$

$$\frac{X_{test}}{X_{control}} = 2^{\Delta\Delta C_T} = 2^{(C_{T,X} - C_{T,R})_{control} - (C_{T,X} - C_{T,R})_{test}}$$

Where $C_{T,\,X}$ is the threshold cycle of the gene of interest and $C_{T,\,R}$ is the threshold cycle of the endogenous reference gene. The threshold cycle (Ct) indicates the fractional cycle number at which the amount of amplified copies reaches a fixed threshold. The relative quantification of the virus present in the test samples was calculated using the Ct values obtained and analysed for level on inhibition by shRNA constructs in BHK21 cells.

[III] RESULTS

3.1. 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 for six constructs using vector specific primers OL408, OL559 which corresponds to the sequences within the vector. The clones which produced a specific band of around 270bp were considered as positive clones. Selected positive clones were confirmed again by PCR of isolated recombinant plasmid DNAs (figure 1). The plasmid DNAs of six positive clones and the vector were digested with Pst1 restriction enzyme (figure2). Release of 340bp fragment indicates that the clones are positive 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. DNAs from positive clones were sequenced with forward and reverse primer and confirmed for insert.

3.2. Transient cellular transfection in BHK-21 cells with combinations of shRNA producing cassettes

BHK_{21} cell lines were transfected with 12 combinations of shRNA producing cassettes of VP1, VP3 and VP6 genes using Lipofectamine 2000 in 24 well plates as per standard protocol. The transfectd cells were further infected with BTV1 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 indicated that the DNA constructs were non toxic to the cell.

3.3. **Effect of combinations of shRNAs on inhibiting replication of BTV-1**

Transfection was carried out with combinations of two shRNAs (total 12 combinations) using Lipofectamine 2000 in 24 well plates as per standard protocol. BTV-1 serotype was used for infection at 100 $TCID_{50}$ to the transfected cells at 8h of pt and harvested at 48h of p.i. and studied for inhibition of virus replication.

Considerable inhibition (up to 4-6 log difference in titre with control) on replication of BTV-1 was observed when the cells were transfected with combinations of shRNA constructs. The combination of VP1shRNA2+VP6shRNA1 showed a titre of $_{10}$Log 4 when the virus control titre was $_{10}$Log 10.3. Only four combinations out of 12 showed a titre value higher than $_{10}$Log 5 even though the value was less than control. They were VP1shRNA1+VP3shRNA1, VP1shRNA1+VP3shRNA2, VP1shRNA2+ VP6hRNA2, VP3shRNA1+VP6shRNA1 given titres $_{10}$Log 5.75, 6.25, 5.69 and 6 respectively. Other combinations were showed titres between $_{10}$Log 4 and $_{10}$Log 5 (table1).

3.4. **qRT PCR of cDNA from cells transfected with combinations of shRNAs to know the inhibition of virus replication**

The cells transfected with combinations of shRNA constructs were infected with BTV1 serotype and harvested 48h p.i. The Ct values of these samples indicated that there was inhibition of replication by all combinations of shRNAs. The Ct value (table1) obtained for virus control was 22.44. The viral RNA from cells transfected with combination of VP1shRNA1+VP3shRNA1, VP1shRNA1+VP3shRNA2, VP1shRNA1+VP6shRNA1, VP1shRNA1+VP6shRNA2, VP3shRNA1+VP6shRNA1, VP3shRNA2+VP6shRNA1 and VP3shRNA2+VP6shRNA2 showed Ct values higher than 26 which indicated a good inhibition of replication.

Sl. No	Samples	$_{10}$Log titre of Virus per 100µl of sample	Ct values
1	**VP1shRNA1+VP3shRNA1**	5.75	28.19
2	**VP1shRNA1+ VP3 shRNA2**	6.25	26.11
3	**VP1shRNA2+ VP3shRNA1**	4.36	23.76
4	**VP1shRNA2+ VP3shRNA2**	4.64	23.34
5	**VP1shRNA1+ VP6shRNA1**	5.0	27.58
6	**VP1shRNA1+ VP6shRNA2**	4.5	26.51
7	**VP1shRNA2+ VP6shRNA1**	4.0	24.13
8	**VP1shRNA2+ VP6shRNA2**	5.69	25.99
9	**VP3shRNA1+ VP6shRNA1**	6.0	26.39
10	**VP3shRNA1+ VP6shRNA2**	4.58	25.23
11	**VP3shRNA2+ VP6shRNA1**	4.36	27.69
12	**VP3shRNA2+ VP6shRNA2**	4.38	26.96
13	**Virus control 48**	10.3	22.44

TABLE 1: Virus titre in cells transfected with combinations of shRNAs and infected with BTV 1 serotype at 100 $TCID_{50}$(48hp.i.) and Ct values on qRT PCR

[IV] DISCUSSION

Bluetongue virus is the well understood type virus of Orbivirus group in Reoviridae family. The function and structure of each protein encoded by 10 double stranded RNA segment have been well defined [16]. While the serotype specific VP2 and VP5 help in virus attachment and entry, the other proteins namely VP1, VP3, VP7 and VP6 have various other functions in virus replication, assembly and release. VP1 an RNA dependent RNA polymerase is one of the most important proteins required for viral replication [2]. While VP3 helps in assembly of viral proteins and interacts with internal minor proteins, VP6 binds to ssRNA and dsRNA with helicase function [17].

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. shRNAs synthesised in the nucleus of cells are further processed and transported to the cytoplasm to get incorporated into the RISC complex [18] where they bind to complementary mRNA sequences. Once bound, the target mRNA is cleaved and is no longer functional and thus the specific protein production is prevented. After efficient introduction of RNAi effectors to target cells, the expression of a protein of interest is reduced by degrading the targeted mRNA. As RNAi degrades target mRNA, not protein, the stability (half life) of target protein is an important factor determining the gene silencing effect, which correlates with the amount of target protein [19].

The cell line used for BTV propagation and maintenance was BHK_{21}, as the virus was well adapted to this cell line. The siRNAs and corresponding hair pin insert oligos were designed using the "siRNA Wizard" software (Invivogen), because the vector was developed by Invivogen. The psiRNA vector used in the study was found to be suitable for the work which had a Bbs1/Bbs1 restriction sites (5'---GAAGAC $(N)_2$↓------3' and 3'---CTTCTG$(N)_6$↑---5'). Although these sites are recognised by the same enzyme, they are different avoiding self ligation of the plasmid, and hence the chance of generation of false positives on transformation is minimized. Maximum reduction in FMD virus titres was seen when two to three constructs of siRNA were used together rather than individually [20-21]. These researchers have also suggested that by targeting multiple sites in a conserved gene it is possible to enhance the silencing effect of gene expression up to 100 %. Kim *et al*. [22] reported the use of multiple short hairpin RNA (shRNA) expression systems to inhibit foot and mouth disease virus (FMDV) replication in BHK21 cells.

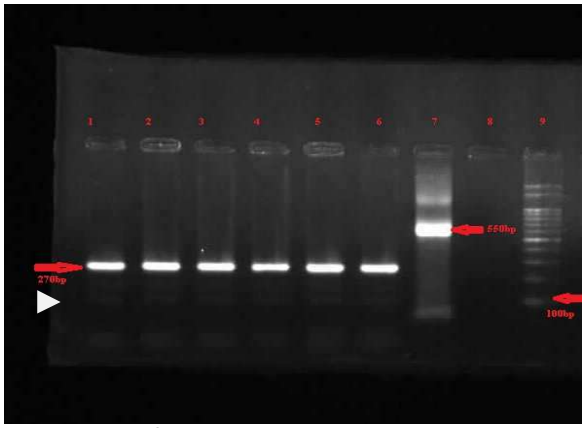

Fig: 1. PCR of positive clones
Lane 1 – 6 positive clones showing 270bp band,Lane 7 – psiRNA vector showing amplicon of 550bp,
Lane 8 – Non template control, Lane 9-100bp DNA ladder

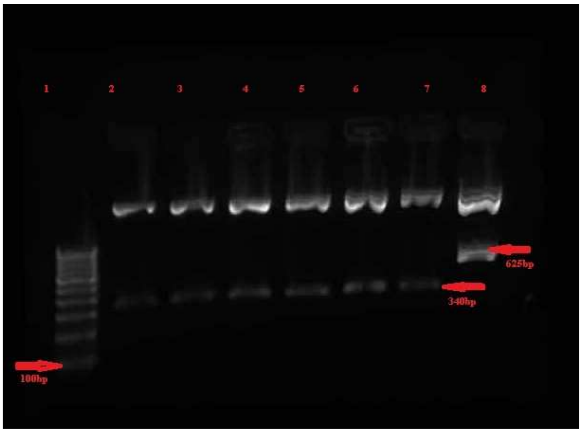

Fig:2. Pst1 digestion of positive clones
Lane 1 - 100bp DNA ladder
Lane 2-7 positive clones releasing 340bp fragment,
Lane 8 – psiRNA vector releasing 625 bp fragment on Pst1 digestion

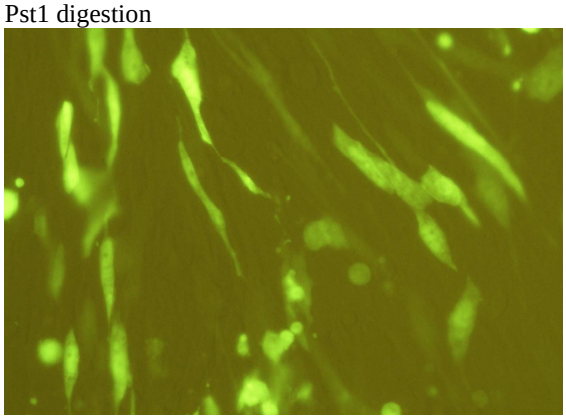

Fig:3. Fluorescence given by BHK21 cells transfected with pcDNA GFP indicating the efficiency of transfection

[V] CONCLUSION

This work is the first report of the use of combinations of shRNA towards the control of BTV. Inhibition of virus replication was observed when combinations of shRNA producing cassettes was used for transfecting the cell to BTV1 serotype at 100$TCID_{50}$ infection and 48h p.i. *in vitro* in BHK21 cells. Knocking down of VP1, VP3 and VP6 genes has given good results for inhibition of virus replication. Further study will be required to determine whether such treatment protect against BTV infection *in vivo*.

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