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CRISPR-Cas9 mediated genome editing of bovine herpes virus with DsRed gene insertion: A novel approach for viral labeling and tracing

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Abstract
Genome editing using CRISPR/Cas9 technology offers several advantages over conventional methods of gene-editing. The method is cost-effective, time saving and highly efficient. The technology has been successfully applied for genome editing of various human and animal DNA viruses for understanding viral replication, pathogenesis, host-virus interaction and generation of viral mutants. In present study we applied this technology for editing of bovine herpesvirus-1(BoHV-1) genome. Plasmid based expression system was used for the delivery of single guide RNA (sgRNA) and targeting of the non-essential gene (US8 coding for glycoprotein E) of the virus. In order to trace the mutant virus, homology directed repair (HDR) method was used. A reporter gene (DsRed) producing fluorescence was inserted at the site of mutation. The fluorescence was detected after third passage of the mutant virus in MDBK cells. The method appears to be promising for tracing of virus mutant from wild-type virus population which is a challenging step in genome editing using CRISPR/Cas9 technology.

Keywords: CRISPR/Cas9, BoHV-1, sgRNA, PX4549, DsRed, HDR, fluorescence

Introduction
Clustered regularly interspaced short palindromic repeats associated with protein nuclease 9 (CRISPR-Cas9), now a well-known genome editing technology was actually derived from bacterial adaptive immune response to invading DNA viruses. This system utilizes a nuclease, Cas and a guide RNA segment that directs the nuclease to a specific target to cut and produce a double-stranded break (DSB) upstream of the protospacer adjacent motif (PAM). In host cells, repair of such DSB can occur either by non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathway (Tang *et al*., 2021) [14]. As of now, two classes and six types of this system have been reported (Mougiakos *et al*., 2016; Makarova *et al*., 2020) [9, 8]. Among these type II system with Cas9 from *Streptococcus pyogenes* (SpCas9) is most commonly used in various field of biotechnology, bioengineering, and translational research. This system was first utilized for genome editing in mammalian and human cells in 2013. Since then, because of its faster, more accurate, and more efficient mode of action as compared to previously existing gene editing technologies, it has become a widely used method for gene editing in present times. In virology, this system has proven very useful in studying virus gene function, host-virus interaction, construction of virus mutants, and inhibition or activation of virus replication. This technology has been successfully applied for genome editing of various DNA viruses including adenovirus, herpes simplex virus type 1, pseudorabies virus, vaccinia virus, Epstein–Barr virus, guinea pig cytomegalovirus, herpesvirus of turkey, and duck enteritis virus (Bi *et al*., 2014; Suenaga *et al*., 2014; Xu *et al*., 2015; Yuan *et al*., 2015; Bierle *et al*., 2016; Peng *et al*., 2016; Tang *et al*., 2016; Zou *et al*., 2017; Chang *et al*., 2018; Atasoy *et al*., 2019) [3, 13, 16, 17, 4, 11, 15, 18, 6, 1]. CRISPR-Cas9-based editing has also been applied as an antiviral strategy by inhibiting the replication of many human and animal viruses including BoHV^{-1}. Bovine herpesvirus-1 belongs to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae* under the family *Herpesviridae* (Chatterjee *et al*., 2016) [7]. BoHV^{-1} is an important viral pathogen causing respiratory and reproductive disease in cattle and buffaloes. The infection causes huge economic losses to the livestock sector worldwide. The genome of bovine herpesvirus has many essential and non-essential genes that can be altered to generate virus mutants and to study gene functions and pathogenesis of the virus. The non-essential gene, US8 has been a popular target of deletion for the development of gene-deleted marker vaccines against the virus.

In the present work, we targeted a non-essential gene of the virus (US8 gene encoding envelope glycoprotein E) using CRISPR/Cas9 technology. The plasmid-based expression system was used for the targeting of the US8 gene. To trace the mutant virus a fluorescent reporter gene (DsRed) was inserted at the mutation site using homology-directed repair (HDR) method.

Materials and Methods

Cell culture, virus propagation and isolation of viral DNA

Madin Darby Bovine Kidney (MDBK) cells maintained in DMEM (Himedia, India) supplemented with 10% FBS (Gibco, USA) at around 70-80% confluencywas infected with BoHV-1(CAD/BoHV-1/1984) from the virus repository of the laboratory. After the virus infection, the media was replaced with DMEM containing 2% FBS, and cells were incubated at 37ºC in a CO_2 incubator. The virus was harvested after the appearance of complete cytopathic effects (CPE). The viral DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Germany). The virus was confirmed to be BoHV-1 by OIE-recommended real-time PCR (OIE Terrestrial manual, 2018) [10].

Construction of CRISPR/Cas9 sgRNA plasmids

For targeting of US8 gene, different pairs of partially complementary oligonucleotides, termed as top and bottom oligos were synthesized using a web-based tool (http://www.rgenome.net/cas-designer/). An Indian isolate BoHV-1 (accession no.KY215944) was taken as a reference genome from NCBI for the purpose of guide RNA designing. The oligos were cloned into Cas9 expression vector pSpCas9(BB)-2A-Puro (or simply PX459) (Addgene), following the protocol described by Ran *et al*., 2013[12]. The cloned sgRNAs were confirmed using colony PCR and further by sequencing.

Editing of US8 gene using plasmid-based expression system

The MDBK cells were seeded in a 4-well cell culture dish (SPL, India). After obtaining appropriate confluency of about 70-80%,cells were transfected with PX459 plasmid containing sgRNA targeting US8 gene using liposomal transfection reagent DOTAP (Roche, Germany) as per the manufacturer's instructions. After 3-4 hrs of transfection, BoHV-1 infection at an MOI of 0.01 was given and cells were incubated at 37 °C in a CO_2 incubator for 24 hrs. A wild-type virus control, neat plasmid control and a cell control were also included.

Detection of mutation in US8 gene

The viral DNA was extracted from the harvested virus pool. The extracted DNA was subjected to PCR using primers specifically designed for the amplification of the CRISPR/Cas9 targeted region of US8 gene (US8 fwd.: 5'CGTGTGTCTTGGTTTCTGCG 3' and US8 Rev: 5'GAAGACCGTGTCGACCGAAG 3'). The mutation was confirmed using high percentage gel electrophoresis (Bhattacharya and Meir, 2019) [2] and T_7 endonuclease assay.

Designing of repair template for HDR

For insertion of DsRed gene at the mutation site of US8 gene, plasmid-based repair templates consisting of homology arms (sequence present at right and left flanking sides of the CRISPR/Cas9 target site) were used. Gene coding for red fluorescent protein DsRed was amplified from pDsRed plasmid (Takara) using primers DsRedF (5' ATGGCCTCCTCCGAGGACGTCATCAAGG 3') and DsRedR (5' GGAACAGGTGGTGGCGGCCCTCGGCGC 3'). Another pair of primers was designed to insert the reporter gene DsRed into the N-terminal of US8 gene of BoHV-1. Overlap PCR was done for the amplification of the repair template. The left arm (gEL), DsRed and right arm (gER) were separately amplified, and gel extracted using Qiagen gel extraction kit. Then two separate reactions were set up to assemble LHA and DsRed first, followed by assembly of DsRed and RHA. The products of both reactions were gel extracted and re-amplified to obtain full-length repair template (gEDsRed) using overlap primers.

The full-length repair template (gEL+DsRed+gER) was gel extracted, quantified and ligated into pJet1.2 vector using Clone JET PCR cloning kit (Thermo Scientific, USA) as per the manufacturer's protocol (Fig.1). The ligation mixture was transformed into *E. coli* DH5α cells following $CaCl_2$ transformation method. The positive colonies were selected by screening by colony PCR and positive clones were further confirmed by sequencing.

Co-transfection of DsRed insert and gEPX459 plasmid for virus labeling and tracing

The gEL+DsRed+gER+pJet1.2 plasmid was linearized near the distal end of right homology arm by digestion with *Xho* I enzyme (Promega). The linearized plasmid was gel purified and quantified, followed by transfection of 500ng of linearized plasmid along with 500ng of PX459 plasmid into MDBK cells.

Confirmation of DsRed insertion into the BoHV-1 genome by fluorescent microscope

The DsRed fluorescence was checked by visualizing the transfected cells through fluorescent microscopy. The transfected cells were further passaged for two generations and the DNA was extracted to check the presence of DsRed gene insertion.

Results

Generation of sgRNA plasmids and construction of US8 deleted BoHV-1

The synthesized sgRNA oligos was cloned into PX459 vector and positive clones were confirmed by amplification of ~250 bp (Fig.2) followed by nucleotide sequencing. DNA extracted from BoHV-1 virus treated with the gEsgRNA+PX459 plasmid was used as template for amplification of the target region in the BoHV-1 genome. Multiple bands were visible upon resolution of amplified product on high percentage (6%) agarose gel electrophoresis along with the wild-type virus band. The multiple amplicons indicated the presence of mutant population along with the wild-type virus population. T_7 endonuclease assay further confirmed the presence of mutation.

Repair template design for HDR

The overlap PCR using gEL (789bp), DsRed (678bp) and gER (648bp) resulted in 1467 bp gEDsRed left homology arm (gEDsRedLHA-1467bp) and gEDsRed right homology arm (gEDsRedRHA-1326 bp). These left and right homology arms were further amplified together by overlap PCR to generate full length repair template of 2793 bp and was named as gEDsRed 2793bp repair template (Fig.3).The gEDsRed repair

template was ligated into pJet1.2 vector and linearized using XhoI (Fig.4).

Tagging of virus with DsRed

Both the plasmids were mixed (500 ng of each) and transfected into the MDBK cells using DOTAP reagent. After 3-4 hrs of transfection, BoHV-1 infection was given (MOI=0.01). The cells were harvested after appearance of complete cytopathic effects. The harvested virus was passaged three times. A schematic for co-transfection is depicted in Fig.5.The presence of red fluorescence in the third passage revealed the insertion of the DsRed in the US8 region of BoHV-1 genome.

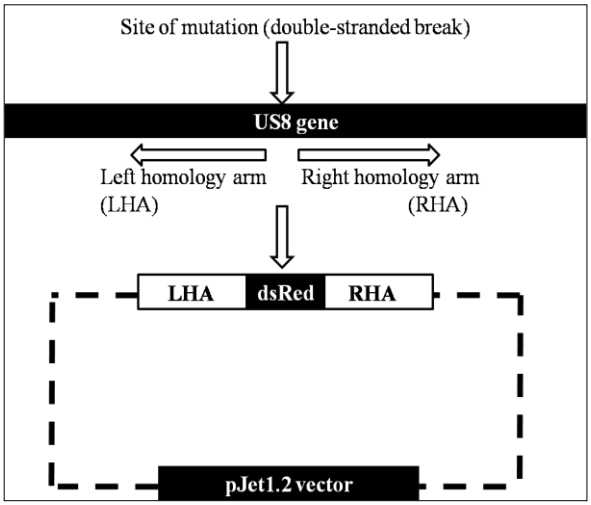

Fig 1: Schematic representation of development of DsRed repair template and ligation into pJet1.2 vector

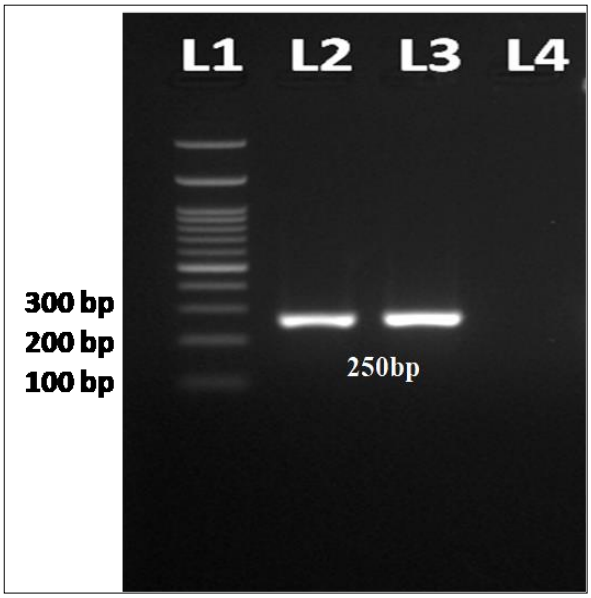

Fig 2: Screening of transformants by colony PCR, using human US6 sequencing primer as forward primer and specific bottom oligo as reverse primer. Desired product of around 250 bp was amplified: L1: 100 bp marker, L2 & L3: ~250 bp from positive colony of sgRNA cloned plasmid, L4: NTC (non-template control)

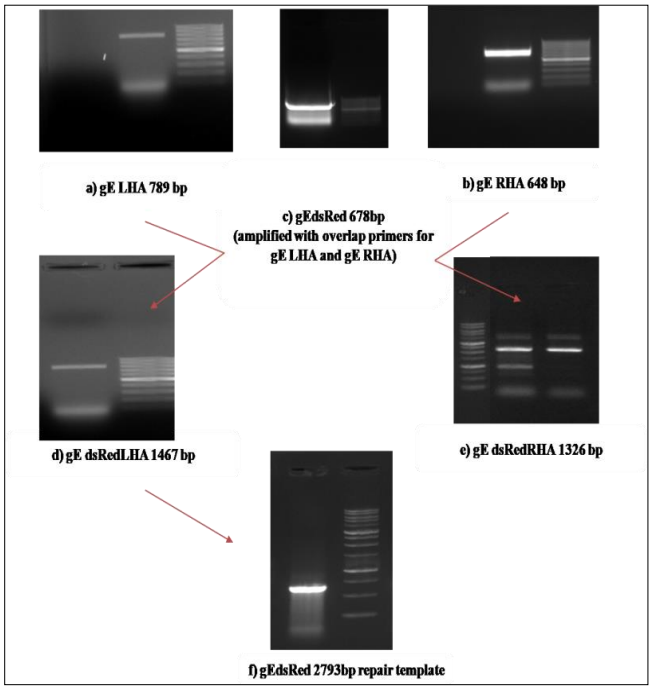

Fig 3: Overlap PCR for the generation of repair template for CRISPR/Cas9 mediated homology directed repair (HDR) for US8 targeted BoHV-1. LHR, DsRed and RHA of 789bp, 678bp and 648bp respectively, were amplified from wild-type BoHV-1 DNA (a,b,c). gEDsRedLHA 1467 bp (d) was amplified by overlap PCR using gel extracted a) and c) as templates whereas gE DsRedRHA 1326 bp (e) was generated by overlap amplification of b) and c). The full length repair template gEDsRed 2793 bp (f) was generated by overlap PCR using d) and e) as templates

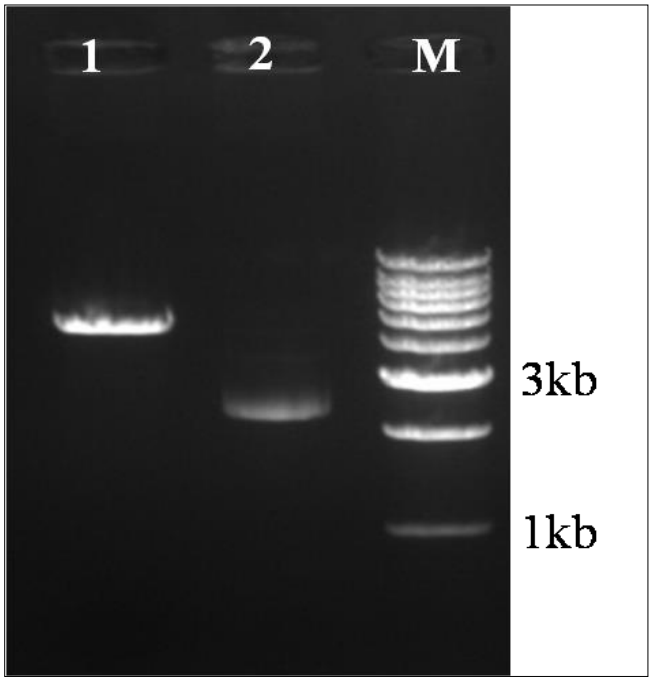

Fig 4: Agarose gel electrophoresis showing linearized pJet1.2 g EDsRed. Lane1: pJet1.2gEDsRed linearized with *XhoI*. Lane2: Uncut pJet1.2gEDsRed. M lane: 1k bp DNA ladder

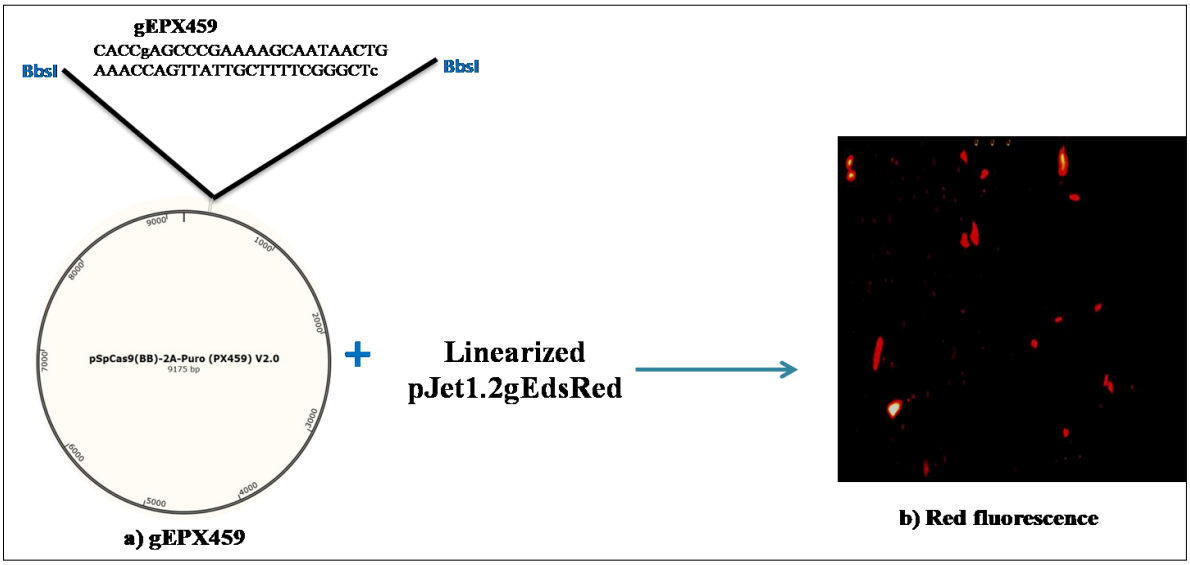

Fig 5: Co-transfection of gEPX459 and linearized pJet1.2gEDsRed into MDBK cells for CRISPR/Cas9 mediated homology directed repair (HDR) in US8 targeted BoHV-1. a) Represents the PX459 vector with US8 sgRNA construct (gEPX459) that was mixed with linearized pJet1.2gEDsRed and transfected into MDBK cells. b) Shows post transfection image of MDBK cells with detectable DsRed fluorescence.

Discussion

CRISPR/Cas9 technology is increasingly becoming popular for genome editing of viruses. A crucial step after gene editing is the segregation of the mutant virus from the wild-type virus population. Tagging with a reporter gene, for example a fluorescence producing gene at the mutation site can be used to fish out the mutant virus. A reporter gene (DsRed) was inserted at the mutation site (US8 gene) of BoHV-1 using homology-directed repair method (HDR). For precise repair of the double-stranded break caused by the Cas9, a repair template (gEDsRedpJet1.2) which provided the homology on both sides of the target sequence of US8 gene was constructed using overlap PCR. This construct having DsRed gene was co-transfected with the gEPX459vector for the insertion of DsRed gene into the BoHV-1. The presence of fluorescence at the third passage revealed the successful insertion of DsRed into the BoHV-1.Fluorescence-activated cell sorting (FACS) can be used for further sorting of the transfected cells (Challagulla *et al*., 2021) [5]. A great advantage of inserting any reporter gene is the visualization of the mutant virus under the fluorescent microscope. The reporter gene when inserted at any specific gene of the virus can give valuable insight on the role and function of that particular gene in the replication and pathogenesis of the virus. A mutation in the non-essential gene of BoHV-1 can be used for the development of a live-attenuated vaccine candidate. A live-attenuated vaccine contains an avirulent virus that does not cause the disease but triggers a protective immune response in the animal. The insertion of a fluorescent gene can be helpful in studying the kinetics of the mutant vaccine candidate *in vitro* and *in vivo* which further aids in the characterization of the vaccine candidate. The fluorescent gene can be used to track the spread and fate of the virus *in vivo* for further research purposes.

Conclusion

CRISPR/Cas9 is now increasingly being employed as a genome editing tool for understanding the virus-host interactions, virus gene-function and pathogenesis of different viruses. It is a quick and economical method for gene knock out and knock in studies. In the present study successful knock out of gE gene followed by insertion (knock in) of a fluorescent gene in BoHV-1 was done using HDR strategy. The insertion of a fluorescent gene is helpful in tracing the mutant virus which is a promising new development that has the potential to improve the understanding of the virus and to develop more effective vaccines and treatments against the virus.

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