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## Research Article

**Keywords:** Bovine herpesvirus-1 (BHV-1), CRISPR/Cas9, Antiviral strategy, Gene editing

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## CRISPR/Cas9 assisted gene targeting efficiently inhibits bovine herpesvirus-1 replication

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### Abstract

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/ CRISPR associated protein (cas) are now being accepted as a highly specific method of gene editing. Among many other applications, CRISPR/cas has an immense potential to be used as antivirals. In this study, we successfully demonstrated CRISPR/Cas9 mediated inhibition of Bovine Herpes virus -1 (BHV-1) replication. BHV-1 causes economically important diseases in bovines with establishment of latency. Six essential genes and one non-essential gene of BHV-1 were targeted to assess the impact on virus replication. Inhibition of UL52, circ, and UL27 genes showed promising results, whereas the other three genes US6, UL18, and UL34 resulted in lower level of inhibition. Non-specific gene editing in host and virus was *in-silico* evaluated and was demonstrated by inhibition of virus induced apoptosis. Successful editing of one viral non-essential gene without any alterations in virus replication demonstrated the potential of CRISPR/Cas9 in replicating viral genome. Complete abrogation of virus replication was observed transiently (~24 hours post-transfection/hpt) when transfected with short lived *in-vitro* transcribed sgRNAs. Whereas, under constant expression of sgRNAs through plasmid, complete inhibition of virus replication was observed till ~72 hours post-infection. Complete inhibition of replication was also observed with *in-vitro* transcribed sgRNA when booster dose of sgRNA was transfected at 24hpt. It has been speculated that

constant expression with plasmid based delivery may result in off-target activity which can be ruled out with short lived in-vitro transcribed sgRNA.

## **Keywords**

Bovine herpesvirus-1 (BHV-1), CRISPR/Cas9, Antiviral strategy, Gene editing

Infectious diseases are one of the biggest threats to the livestock and poultry industry around the globe. Arguably, diseases of viral aetiology are considered crucial among all infectious diseases as they spread rapidly across the boundaries and results in serious socio-economic and public health consequences [1]. Large-scale mortality, drastic reduction in farm output, zoonotic potential, and trade embargo are some of the major concerns related to the viral diseases [2]. Control and eradication of viral pathogens are essential towards achieving global food security [3]. With a lack of effective antiviral drugs, control efforts rely mainly on vaccination and biosecurity measures. Some diseases have been controlled and eradicated whereas many important viral diseases are still endemic and continue to cause huge economic losses despite several vaccination-based efforts. Bovine herpesvirus-1 (BHV-1) is one such example that is endemic in many countries. BHV-1 belongs to the *Alphaherpesvirinae* subfamily of the *Herpesviridae* and cause diseases in domestic and wild cattle. During the primary infection BHV-1 causes abortion, infectious bovine rhinotracheitis (IBR), and infectious pustular vulvovaginitis (IPVV), after which the cattle become a latent carrier [4]. This virus establishes latency mainly in ganglionic neurons where it expresses only a few viral genes and remains inside the host without being noticed by the host's immune response and such latent carriers are the main source of disease dissemination [5-7]. The available vaccines prevent the clinical impact of the disease but not virus infection and latency establishment [4]. Currently, no therapeutics is available for BHV-1 infected/carrier animals.

Lately, CRISPR/Cas9 gene-editing technology has emerged as a powerful tool for RNA-guided specific genome modifications, which has the potential to develop new generation therapeutics. CRISPR/Cas9 system has been tested for its therapeutic potential (*in-vitro* and *in-vivo*) for various viruses (reviewed in [8]), either through direct disruption of the viral genome or by hampering the host factors helping in the virus replication [8]. To explore

a similar strategy for BHV-1, the present study was designed to evaluate the efficiency of the CRISPR/Cas9 system to limit BHV-1 replication.

The essential and non-essential genes required for *in-vitro* replication of BHV-1 were selected as described by Robinson and co-workers [9]. Six essential genes spread across the three temporal expression zones *viz.* immediate-early (IE), early (E), and late (L) genes were targeted to achieve maximum inhibition of replication. Glycoprotein E (gE) gene of BHV-1 which is not essential for *in-vitro* replication was also targeted with CRISPR/Cas9 to evaluate specific genome editing and effects on virus replication. The selected genes and their functions are detailed in table 1. BHV-1 subtype 1.1 was revived in Madin-Darby Bovine Kidney (MDBK) cells maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. Cells were observed for cytopathic effects (CPE), the virus was titrated using the end-point dilution method [10] and stored at -80°C till further use.

The single-guide RNA (sgRNA) targets for each selected gene, were designed using the CRISPR RNA guided engineered nucleases (RGENs) tool (<http://www.rgenome.net/cas-designer/>) and the ones with the highest score for specificity (least/no off-targets) within bovine and BHV-1 genome were selected (table 1). To further ensure the specificity, *in-silico* analysis of sgRNA targets was performed using the web server of Cas-OFFinder (<http://www.rgenome.net/cas-offinder/>). The primers for the synthesis of the sgRNA DNA template for each selected gene were synthesized commercially (Eurofins, India). sgRNA DNA templates were PCR assembled and confirmed by 2% agarose gel electrophoresis (AGE). The confirmed templates were *in-vitro* transcribed to sgRNA, using a commercially available kit (GeneArt precision gRNA synthesis kit, Invitrogen) as per the recommended protocol.

MDBK cells were seeded in 24 well plate and at 60-80% confluency, cells were infected with BHV-1 (1 MOI). 3-4 hours post-infection (hpi), 125 ng of *in-vitro* synthesized sgRNA was complexed with 625ng of Cas9 enzyme (Invitrogen) to form RNP complex and transfected into BHV-1 infected MDBK cells using CRISPRMAX transfection reagent as per the manufacturer's recommendations. Virus and mock-infected cell controls were included and the plates were regularly observed for the appearance of characteristic CPE.

Along with RNP-based expression, the sgRNA target sequences were also expressed *via* U6 promoter-driven PX459 vector, following the protocol described by Ran and co-workers [11]. Briefly, sgRNA target sequences were cloned in PX459 vector, transformed in the DH5 $\alpha$  strain of *E. coli*. and the plasmids were extracted using an endotoxin-free plasmid extraction kit (Qiagen plasmid midiprep kit). Similar to RNP based expression method, the MDBK cells were seeded and transfected with plasmids (500ng/well in 24 well plate) cloned with each sgRNA target using p3000 reagent (Thermo, USA) as per manufacturer's instructions. For plasmid-based transfection, BHV-1 infection (1MOI) was given 12-14 hours post-transfection (hpt).

BHV-1 infected and mock-infected cells were harvested in triplicates at 06, 12, 24 and 48 hpt for RNP transfected cells and at 72 hpi for plasmid transfected cells. Virus quantification was performed by endpoint dilution method of titration [11]. Plaque-assay was performed for virus quantification and comparison of plaque morphologies of wild type (WT) and CRISPR targeted BHV-1, as described by Baer and Hall [12]. Viral DNA load was quantified by real-time PCR assay using SYBR chemistry. Specific forward primer (TGTGGACCTAAACCTCACGGT) and reverse primer (GTAGTCGAGCAGACCCGTGTC) were synthesized for quantification using plasmid DNA standards as described elsewhere [13]. In order to ascertain the inhibition of virus replication, virus induced apoptosis [15] was estimated using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (Promega, USA) as per the manufacturer's instructions.

T7 endonuclease assay was performed to evaluate gene editing in BHV-1 that was targeted for a non-essential gene (gE), using commercially available kit (GeneArt Genome Cleavage detection kit, Thermo, USA) as per the recommendations. Forward primer (FP: CGTGTGTCTTGGTTTCTGCG) and reverse primer (RP: GAAGACCGTGTCGACCGAAG) were designed to amplify CRISPR targeted region to confirm the insertion/deletion. Bhattacharya and Meir, [14] demonstrated that CRISPR/Cas9 induced insertions/deletions can also be detected by high density agarose gel electrophoresis. Therefore, the amplified (used FP and RP) target gene was also resolved on high density (5%) agarose gel.

Characteristic CPE for BHV-1 appeared after 12hpi and completed by 48hpi (Fig. 1a-d). The mock infected cells did not show any significant changes till 60hpi. Standard growth curve for virus replication was prepared with the data obtained from virus titration and real-time PCR. The RNP-based experiment exhibited significant reduction in CPE up to 24 hpt (Fig. 1e-g). However, complete CPE was observed at 48hpt (Fig. 1h) which indicated that the RNP complex successfully inhibited the virus replication for 24hpt but not beyond. Contrarily, the CPE in non-essential gene treated BHV-1 (Fig. 1i-l) approximated with WT BHV-1. To confirm this observation, percentage inhibition (PI) of replication in CRISPR/Cas9 treated virus relative to un-treated virus (WT) was calculated, by the end-point dilution method of titration, and the findings corroborated the visual changes. More than 60% inhibition (relative to BHV-1 controls) was observed at 24hpt in BHV-1 treated with sgRNA against essential genes (Fig. 2a-g). Our experiment revealed that targeting UL52, circ, and UL27 genes of BHV-1 abrogated the virus replication most effectively (98-99.9%) (Fig. 2a-c), whereas targeting US6, UL18, and UL34, genes resulted in comparatively lower inhibition (68-92%) (Fig. 2e-g). Furthermore, to completely abrogate the virus replication through RNP based platform, two refinements were tried. At first, all the selected essential genes of BHV-1 were simultaneously targeted but only marginal improvement in inhibition of virus replication was achieved with RNP-based multiple targeting over single gene targeting (Fig. 2d). Secondly, in addition to single dose of RNP complex at 6 hpi, a booster dose was given at 24hpt. Complete abrogation of BHV-1 replication was observed when treated with second dose of RNP. Constant expression of sgRNA through vector-based system against essential genes of BHV-1 also completely inhibited the virus replication till 72hpi.

On the other hand, statistically insignificant ( $p>0.05$ ) inhibition was observed in the BHV-1 treated with sgRNA targeting a non-essential (gE) gene (Fig. 2h), which indicates no harmful effects of the components of CRISPR/cas9 system. Viral gene editing was successfully demonstrated by T7 endonuclease assay which resulted in three amplicons (one; parental and two; cleaved products) in the virus population confirming the presence of mutant as well as WT BHV-1 (Fig. 3c). Similar findings were obtained when PCR amplicons were resolved in high concentration (>5%) agarose gel electrophoresis that showed multiple PCR amplicons in CRISPR targeted gE amplified products (mutants/MT) which were absent in the untreated WT BHV-1 (Fig. 3b).

Plaque assay analysis (Fig. 4a-d) revealed that BHV-1 targeted for essential genes produced very few plaques (Fig. 4c) whereas non-essential (gE) gene targeted BHV-1 produced plaques that were smaller in size (Fig. 4d) as compared to WT BHV-1 plaques (Fig. 4b).

Inhibition of virus replication was also demonstrated by TUNEL assay where virus induced apoptosis (Fig. 5b) was reduced in the essential gene targeted BHV-1 (Fig. 5c). On the other hand, negligible changes were observed for a non-essential gene targeted BHV-1 (Fig. 5d).

Over the past few years, specific gene editing by CRISPR/Cas9 has exemplified the possibility for the development of new generation antivirals. In a very short span of time many researchers have demonstrated its use as a potential antiviral strategy in several viruses including hepatitis C virus, human immunodeficiency virus, herpesviruses (herpes simplex virus-1, Epstein-Barr virus, pseudorabies virus), hepatitis B virus, human papillomavirus, etc [16-20]. In this study, we demonstrated the antiviral potential of CRISPR-Cas9 against BHV-1. It was observed that BHV-1 replication is inhibited by a minimum of 60% on targeting any one of the six essential genes evaluated in this study. BHV-1 genes namely UL52, circ, and UL27 were shown as potential targets to impair BHV-1 replication (Fig. 2a-c). Among all the targeted essential genes, UL27 (encodes for glycoprotein B/ gB) could be the most potential single target because it starts one of the earliest contacts with the host cell, since it acts as a ligand for attachment receptors and also has a role in the fusion of virus envelope with the host cell membrane [4]. We also observed that the RNP complex resulted in significant inhibition only up to 24hpt, and the virus replication again peaked after 24hpt (Fig 2a-g). The plausible reason behind incomplete abrogation could be the limited half-life of the Cas9 enzyme which is approximately 20hrs [21]. Complete abrogation of virus replication has been shown for other viruses using vector-based sgRNA/Cas9 delivery system [16-19]. Complete abrogation of BHV-1 using a vector-based delivery system against all the six genes. Although plasmid-based delivery showed promising results in the inhibition of virus replication, but it is speculated that with plasmid system, there are chances of integration in host genome and persistent activity of Cas9 enzyme might lead to non-specific gene editing in virus or host genome [22]. Therefore, RNP platform with a short half-life should be preferred for the development of antivirals. Consequently, we attempted the delivery of the second dose of sgRNA/Cas9 after 24hpt and complete viral replication abrogation was

observed, that was similar to the vector-based delivery system. Non-specific effects of sgRNA/Cas9 based gene editing on virus replication were evaluated by keeping one control (gE gene) which is not essential for virus replication in the experiment. No significant drop in virus titer was observed after targeting gE (Fig 2h). Successful gE gene editing was achieved by CRISPR-Cas9 (Fig 3b). The mutation(s) was confirmed by T7 endonuclease assay indicating the specific nature of genome editing by CRISPR/Cas9 (Fig 3c). Off-target activities of the designed sgRNAs were evaluated *in-silico* and by sequencing the amplified top five off-targets in the host genome wherein we did not find any changes within the amplified regions. Although CRISPR/Cas9 mediated gene editing is highly specific in nature, confirming minimum/no non-specificity is of utmost importance for the development of antivirals, to ensure safety [8].

It is concluded that BHV-1 replication can be inhibited using CRISPR/Cas9 based gene editing. Although constant expression of sgRNA through vector-based system resulted in complete abrogation of replication over RNP-based platform for a longer duration, but the short half-life of the RNP complex has the advantage of eliminating the chances of off-target activity, which is the most crucial concern for developing new generation antivirals. Hence, multiple dosing of short-lived sgRNAs/Cas9 complex may be attempted for complete inhibition of virus with minimum or no off-target activity. However, further studies including host genome-wide screening to detect non-specificity would be required to confirm the specific antiviral potential of CRISPR/cas9 before it is evaluated in the laboratory animals.

## **Declarations**

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**Conflict of Interest/Competing Interests:** All authors declare that there is no conflict of interest concerning funding or any other.

**Availability of data and material:** Data generated during the study is available from the corresponding author on reasonable request.

**Code availability:** Not applicable.

**Authors' Contribution Statement:** GKS, RKS, VKG, and SM designed the work. GKS, RKA, and PD conducted the experiments and wrote the manuscript. SN, RKA, and VC contributed to the reagents, kits, and proofread the manuscript. ARC, BS, KPS, and SMG helped in cell culture and molecular work. All authors approved the manuscript.

**Ethics approval:** This article does not involve any studies with human participants or animals by any of the authors.

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**Fig 1** MDBK cells were infected with BHV-1 (1MOI), 3-4 hrs prior to sgRNA/Cas9 complex transfection. **a-d** represents the BHV-1 controls (no gene-targeted) observed at 6, 12, 24, and 48 hpt for changes in CPE. **e-h** shows inhibition of CPE by sgRNA/Cas9 complex targeted against six essential genes (UL52+ Circ+ UL27+US6+UL18+UL34). CPE changes in non-essential gene (gE) targeted BHV-1(**i-l**)

**Fig 2** CRISPR/Cas9 targeting of essential genes inhibits BHV-1 replication (**a-g**). BHV-1 infected MDBK cells were transfected with sgRNAs against the indicated genes (**a-h**). To assess the percentage of BHV-1 replication inhibition (relative to virus control), viruses were collected at 6, 12, 24, and 48 hours post-transfection (hpt) and serially diluted 10-fold (10<sup>-1</sup> to 10<sup>-8</sup>). Each dilution was replicated six times and the virus titer (TCID<sub>50</sub>/ml) was calculated using Reed and Muench method and (**h**) shows the BHV-1 replication inhibition for a non-essential gene

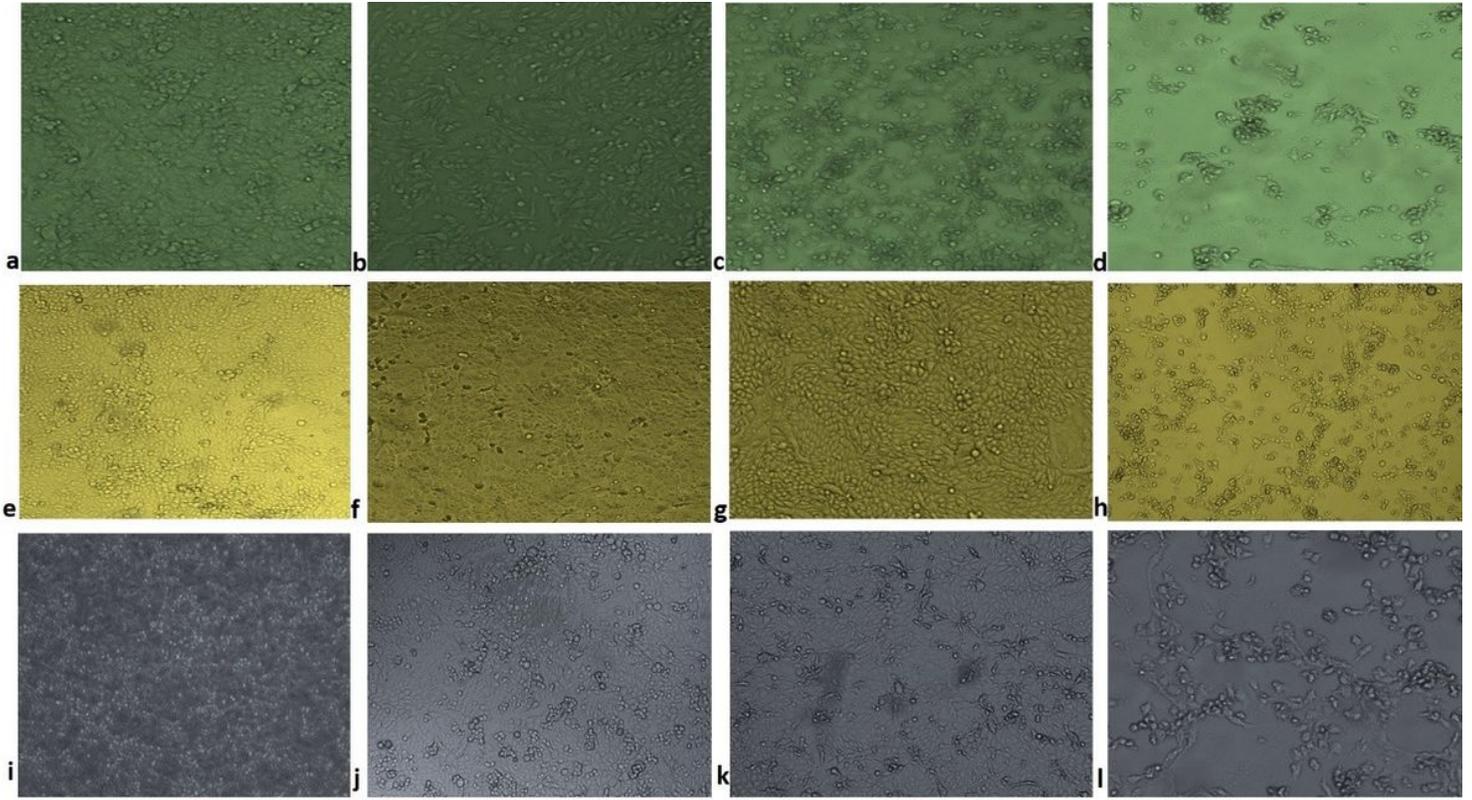
**Fig 3** Detection of gE gene mutants. **(A)** The sequence of PCR amplified region targeting gE gene of BHV-1, including sgRNA target (highlighted yellow). Primer (FP and RP) positions used for amplification are shown (orange arrows) which amplifies an expected 381bp amplicon of wild type (WT) virus **(B)** Gel image showing WT and mutant (MT) amplicons, M denotes 100bp DNA marker. CRISPR/Cas9 targeted region was PCR amplified using primers FP/RP and the PCR products were size separated by high concentration (5%) agarose gel electrophoresis for 80 min. Two amplicons of 381bp (WT) and 400bp mutant (MT) were seen. Heteroduplex bands were visible in mutant BHV-1. **(C)** The same set of primers (FP/RP) were used to amplify 381bp amplicon, after denaturation/re-annealing, samples were treated with

and T7 endonuclease enzyme and were run on a 2% agarose gel. Three bands: 381 (parental/WT), 300bp, and 80bp cleaved bands were seen in the mutated BHV-1 whereas a single band (381 bp) was visible in WT BHV-1

**Fig 4** Reduction in BHV-1 plaques with inhibition of essential genes by CRISPR-cas9 at 24hpi. **(a)** Non-infected cell control **(b)** un-treated BHV-1 (WT) in 10-2 dilution **(c)** BHV-1 treated with pool of sgRNAs against UL52, Circ, UL27, US6, UL18 and UL34 in 10-2 dilution **(d)** BHV-1 treated with sgRNA against non-essential gene (gE) in 10-2 dilution

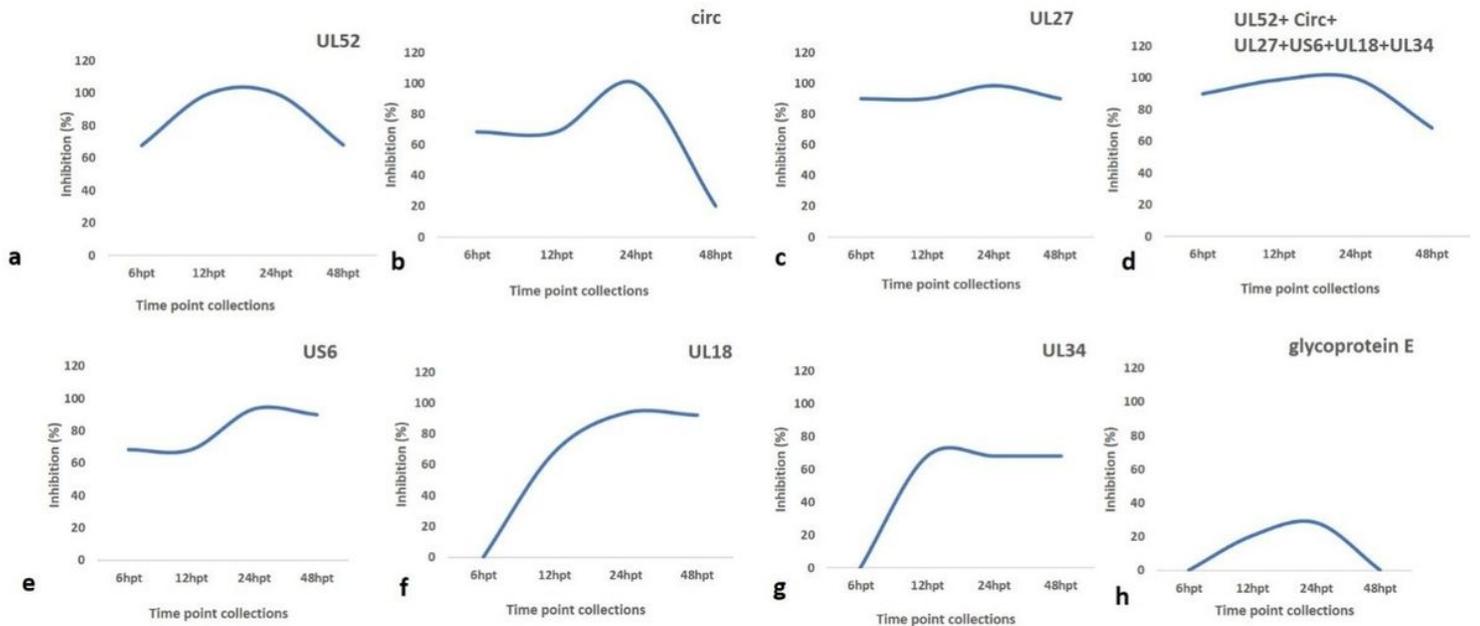
**Fig 5** Inhibition of BHV-1 replication resulted in reduction of virus induced apoptosis in MDBK cells by inhibiting the essential genes of BHV-1. Apoptosis-positive cells were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (uptaking brown color)**(a)** MDBK cell control **(b)** BHVI-1 (WT) control **(c)** CRISPR targeted essential genes **(d)** CRISPR targeted non-essential (gE) gene

# Figures



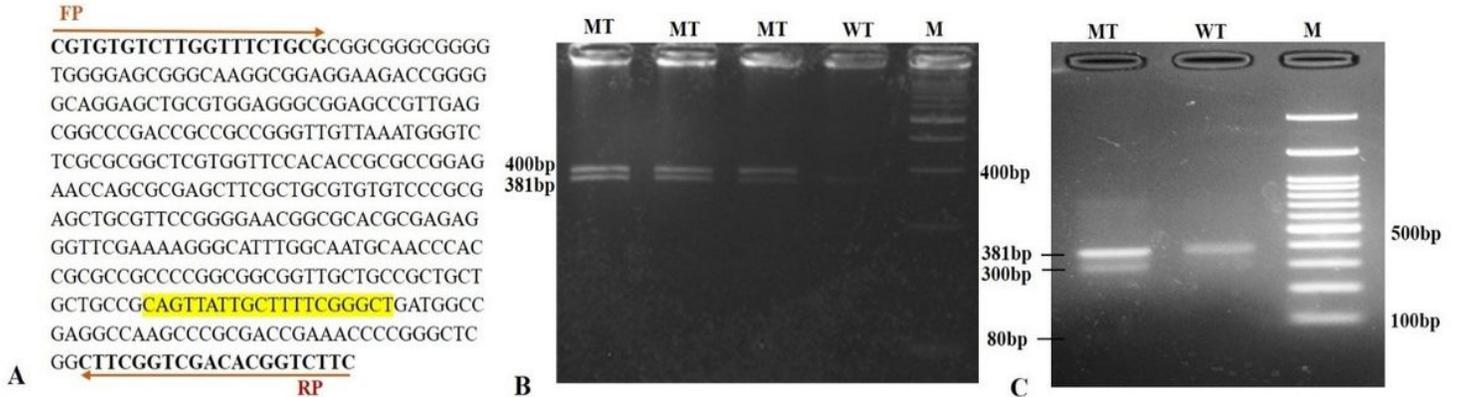
**Figure 1**

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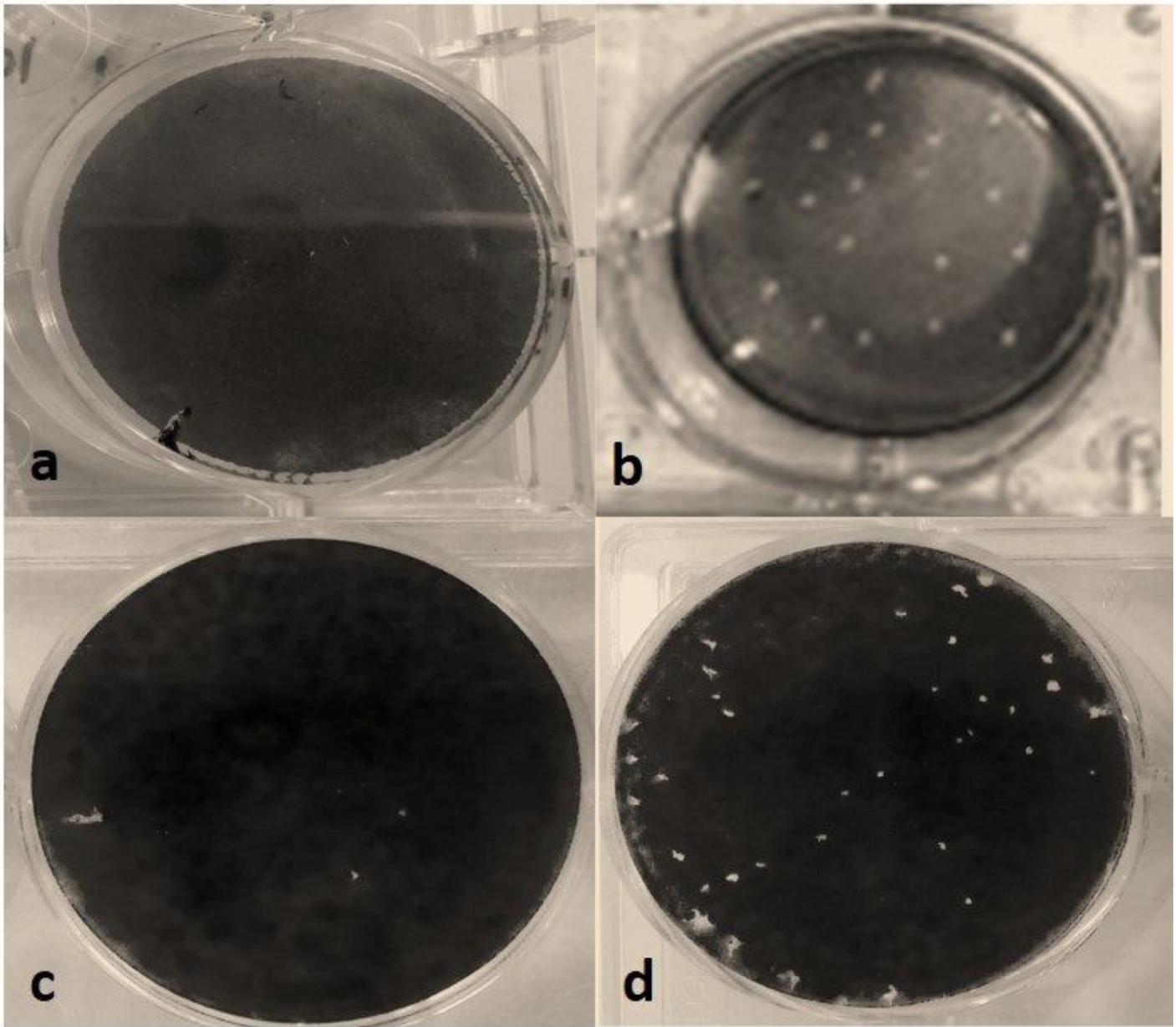
## Figure 2

CRISPR/Cas9 targeting of essential genes inhibits BHV-1 replication (a-g). BHV-1 infected MDBK cells were transfected with sgRNAs against the indicated genes (a-h). To assess the percentage of BHV-1 replication inhibition (relative to virus control), viruses were collected at 6, 12, 24, and 48 hours post-transfection (hpt) and serially diluted 10-fold ( $10^{-1}$  to  $10^{-8}$ ). Each dilution was replicated six times and the virus titer (TCID<sub>50</sub>/ml) was calculated using Reed and Muench method and (h) shows the BHV-1 replication inhibition for a non-essential gene



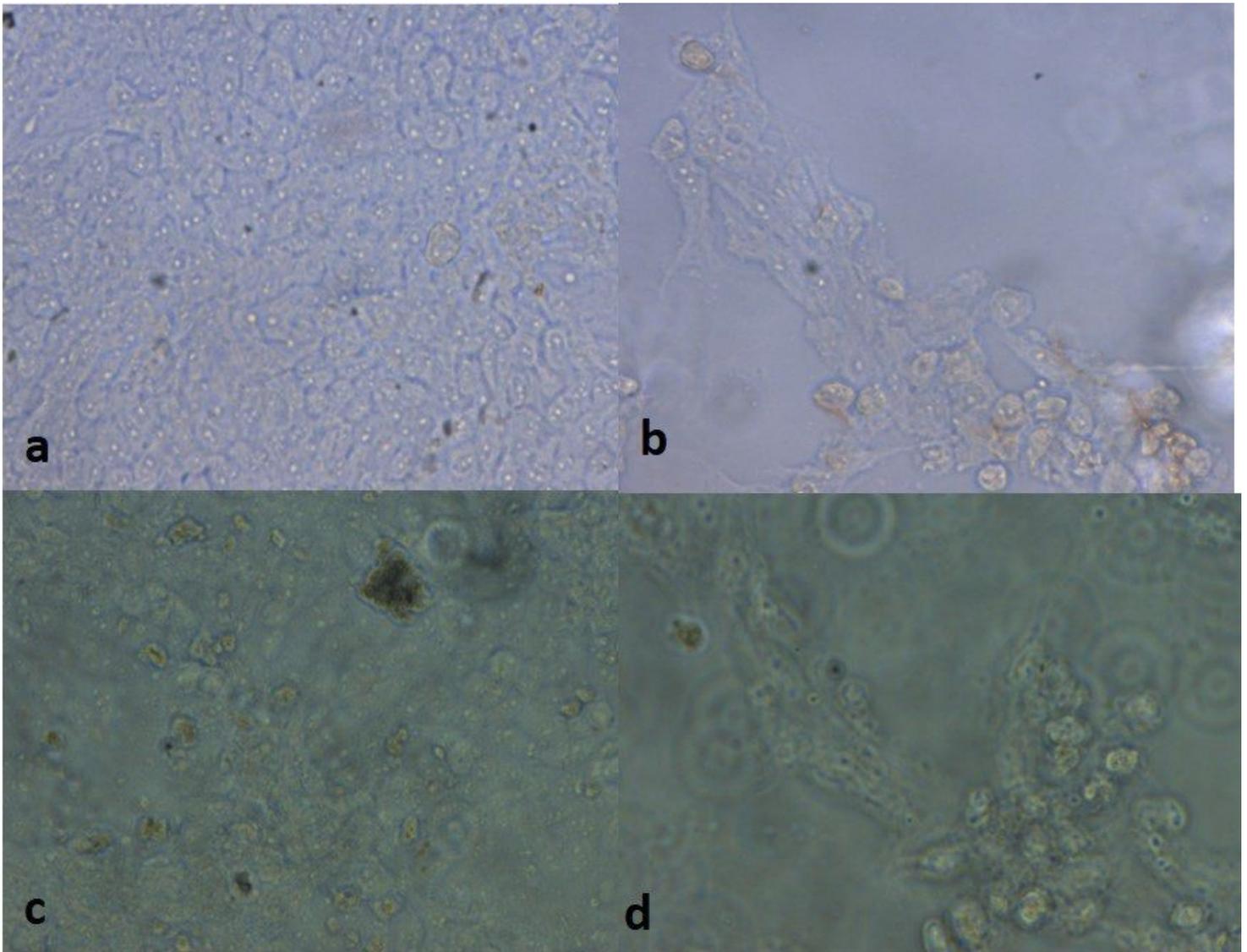
## Figure 3

Detection of gE gene mutants. (A) The sequence of PCR amplified region targeting gE gene of BHV-1, including sgRNA target (highlighted yellow). Primer (FP and RP) positions used for amplification are shown (orange arrows) which amplifies an expected 381bp amplicon of wild type (WT) virus (B) Gel image showing WT and mutant (MT) amplicons, M denotes 100bp DNA marker. CRISPR/Cas9 targeted region was PCR amplified using primers FP/RP and the PCR products were size separated by high concentration (5%) agarose gel electrophoresis for 80 min. Two amplicons of 381bp (WT) and 400bp mutant (MT) were seen. Heteroduplex bands were visible in mutant BHV-1. (C) The same set of primers (FP/RP) were used to amplify 381bp amplicon, after denaturation/re-annealing, samples were treated with and T7 endonuclease enzyme and were run on a 2% agarose gel. Three bands: 381 (parental/WT), 300bp, and 80bp cleaved bands were seen in the mutated BHV-1 whereas a single band (381 bp) was visible in WT BHV-1



**Figure 4**

Reduction in BHV-1 plaques with inhibition of essential genes by CRISPRcas9 at 24hpi. (a) Non-infected cell control (b) un-treated BHV-1 (WT) in 10<sup>-2</sup> dilution (c) BHV-1 treated with pool of sgRNAs against UL52, Circ, UL27, US6, UL18 and UL34 in 10<sup>-2</sup> dilution (d) BHV-1 treated with sgRNA against non-essential gene (gE) in 10<sup>-2</sup> dilution



**Figure 5**

Inhibition of BHV-1 replication resulted in reduction of virus induced apoptosis in MDBK cells by inhibiting the essential genes of BHV-1. Apoptosis-positive cells were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (uptaking brown color)(a) MDBK cell control (b) BHV-1 (WT) control (c) CRISPR targeted essential genes (d) CRISPR targeted non-essential (gE) gene

## Supplementary Files

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- [Table.pdf](#)