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Attenuation of equine herpesvirus 1 through deletion of gE gene and its pathological evaluation in murine model

Bidhan Chandra Bera*, Taruna Anand, Selvaraj Pavulraj, Venkataramireddy Balena, Stephanie Pradhan, Raj Kumar Singh, Bhupendra Nath Tripathi, Nitin Virmani*

ICAR - National Research Centre on Equines, Hisar, Haryana 125 001, India

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Summary. – Equine herpesvirus 1 (EHV1) infection is a global health problem in equines and the virus is responsible for abortions, respiratory disease and myeloencephalitis in horses. Disease management requires proper biosecurity and immunoprophylactic measures. Vaccines strengthening both arms of immunity are essential for proper control and there has been a continuous focus in this area for generation of better vaccines. Here we report construction of bacterial artificial chromosome (BAC) clone of EHV-1 strain Tohana for mutagenesis of the virus and generation of gE gene deletion mutant EHV1. The BAC clone was generated by inserting the mini-F plasmid replacing ORF71 of EHV1 and transforming into *E. coli* for generation of EHV1-BAC. The infectious virus was regenerated from EHV-1 BAC DNA in RK13 cells. To check utility of EHV1-BAC, we have generated mutant EHV1 by deleting the virulence-associated gE gene. The mutant virus (vToHΔgE) showed significantly reduced plaque size without affecting replication efficiency. Pathological evaluation of lesions in BALB/c mice infected with vToHΔgE revealed reduction in clinical signs and pathology in comparison to the wild-type virus. Generation of infectious BAC of EHV1 and its usage in construction of attenuated viruses shows potential of the technology for development of indigenous modified live vaccine for EHV1.

Keywords: equine herpesvirus 1; bacterial artificial chromosome (BAC); mutation; glycoprotein E; vaccine

Introduction

Equine herpesvirus 1 (EHV-1) causes multifaceted infections in equines including abortion storm, respiratory disease and neurological disorder in equines globally. The disease is endemic worldwide in all equine-populated countries including India and infections incur huge economic losses in the equine sector. EHV-1 belongs to the genus Varicellovirus of the subfamily *Alphaherpesvirinae*, has a class D genome consisting of a long and a short unique region (UL and US), both flanked by inverted repeats (TRL/IRL and IRS/TRS) (Telford, 1992; Davison, 2002; Tsujimura *et al.,* 2006). In India, equine abortions are frequently reported due to EHV1 infections, however, the neurologic isolates of EHV-1 have been reported recently in a retrospective study (Anagha *et al*., 2017). The neurological form of the disease, although thoroughly unexplored in India, is one of the most burning problems in other parts of the globe including Germany, UK, US, France, Poland, Argentina, Japan, etc. in recent years (Vissani *et al*., 2009; Smith *et al.,* 2010; Fritsche, *et al.,* 2011; Tsujimura *et al.,* 2011; Stasiak *et al*., 2015). Such an epidemiological shift in the disease pattern is very

*Corresponding authors. E-mails: nvirmani@gmail.com (Nitin Virmani), bcbpatent@gmail.com Bidhan Chandra Bera); phone: +91-1662-275787.

Abbreviations: BAC = bacterial artificial chromosome; CAM = chloramphenicol; CPE = cytopathic effect; dpi = day(s) post-infection; EHV1 = equine herpesvirus 1; EL = primary equine lung cell; gE = glycoprotein E; gfp/GFP = green fluorescence protein; MDBK = Madin-Darby bovine kidney; PK15 = porcine kidney cell line; RK13 = rabbit kidney epithelial cell line; Vero = African green monkey kidney cell line; vToH = EHV1 virus Tohana strain; vToH-BAC = recombinant fluorescence EHV1 virus

much alarming and requires preparedness to control the infection. Moreover, due to the inherent nature of latency associated with EHV1 infection, the long-sought control of the disease primarily depends on the attenuated live virus vaccines because of evoking a longer-lasting immune response (often both humoral and cellular). This has attracted the attention of the world scientific community towards research on deciphering the molecular mechanism governing the pathogenesis, host-pathogen interactions during the disease and the development of new generation of vaccines.

The bacterial artificial chromosome (BAC) technology has opened up new directions in the manipulation of viral genome through the construction of recombinant viruses and subsequent application in deciphering the biology and pathogenesis of herpesviruses (Warden *et al.,* 2011). BACs have been implemented in the manipulation of several human and animal herpesviruses (Brune *et al.,* 2000; Tischer and Kaufer, 2012). The prime advantages of BAC are the stable maintenance of the genome as a clone in *E. coli* with high fidelity of replication and easy introduction of genetic mutations through various mutagenesis strategies including RecA- and Red/ET-mediated recombination methodology (Tischer and Kaufer, 2012). This technology has been successfully implemented in the functional analysis of different genes of herpesviruses in deciphering their role in disease pathogenesis as well as in the development of attenuated live vaccines to combat disease. Among the virulence-associated genes, the gE protein encoded by gene 74 of EHV1 has been shown to be involved in cell-to-cell spread (Mutsumura *et al*., 1998), causing disease pathogenesis. The naturally attenuated KyA strain has inactivated gE gene, which causes attenuation of the virus (Mutsumura *et al*., 1998).

In the present study, we have generated an infectious clone of EHV1 in the form of a bacterial artificial chromosome construct by inserting the mini-F plasmid in place of gene 71 (g71) of the EHV1 genome isolated from severe abortion cases in mare. The EHV1-BAC clone and regenerated virus from the EHV1-BAC DNA were thoroughly characterized by restriction digestion analysis of the genome. Further, the expression of the foreign gene-*gfp* present in the mini-F plasmid was evaluated *in vivo* in mouse lungs. The utility of the EHV1-BAC for generating gene deletion mutant was evaluated by deletion of the virulence-associated gE gene and the mutant virus was characterized *in vitro* and *in vivo* in a mouse model. The gE gene-deleted mutant EHV1 showed reduced plaque size and fewer pathological lesions in the lungs compared to wild-type EHV1 (vToH). The infectious EHV1-BAC clone will serve as a state-of-the-art resource for manipulation of the EHV1 genome for deciphering disease pathogenesis, for the development of deletion mutation-based live attenuated vaccine and also as a possibility of using attenuated virus as vector for delivering the immunogenic proteins of other pathogens in different livestock species.

Materials and Methods

Virus, bacteria and cells. EHV-1 strain Tohana (vToH) isolated from an aborted fetus during outbreaks of abortions in mares in 1996 (Rattan *et al.,* 1998) was used for generation of infectious EHV1-BAC clone and mutant virus. *E. coli* was used for generation and mutagenesis of EHV1-BAC. The RK13 cells were used for propagation and growth kinetics evaluation of viruses. The Vero, PK15, primary equine lung cells (EL) and MDBK cells were used for replication of mutant EHV1 virus. The wild-type and mutant EHV1 viruses were propagated in RK13 cells in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum, penicillin (5,000 U/ml), and streptomycin (5,000 U/ml).

Transfer plasmid construction, transfection and generation of recombinant virus. The transfer plasmid pHA2-g71 was constructed by cloning amplicons of upstream (~2.0 kb) and downstream (2.4 kb) flanking regions of g71 (ORF71) into pUC19 vector and the resulting recombinant plasmid (termed pUC_g71) was subsequently cloned into BAC vector-pHA2 (Adler *et al.,* 2000; Rudolph *et al.,* 2002; a generous gift from Prof. Martin Messerle, Hannover, Germany). The pHA2 vector contains genes for partition viz., sopA, B, C & repE genes; an origin of replication (oriS); the enhanced green fluorescent gene (*gfp*) under the control of the immediate early promoter of human cytomegalovirus (HCMV-IE); *E. coli* xanthine-guanosine phosphoribosyl transferase (*xgpt*) and chloramphenicol resistance gene (*cat*) as selection marker. The confirmed construct of the transfer plasmid was named pHA2_g71.

The purified transfer plasmid (pHA2_g71) was inserted into EHV1 genome by replacing gp2 gene (ORF71) as described earlier (Rudolph *et al.,* 2002). Five to 10 µg of transfer plasmid were transfected into 2×10^6 RK13 cells using polyethyleneimine (PEI) (Polysciences, Inc., USA) @ 1:5 (PEI:DNA) (Ma *et al.,* 2012; Said *et al*., 2017) and incubated at 37°C. The following day, cell culture supernatant was removed, and transfected cells were infected with vToH at a multiplicity of infection (MOI) of 0.1 and observed daily for CPE. The cells were harvested when CPE was advanced and the virus was passaged in RK13 cells. The virus plaques expressing the GFP were picked up and purified by plaque assay for obtaining a homogenous population. The recombinant fluorescent virus was designated as vToH-BAC.

Generation of EHV1-BAC clone and regeneration of the recombinant virus. The circular forms of recombinant virus (vToH-BAC) DNA was isolated by modified Hirt's procedure (Hirt, 1967; Akhmedzhanova *et al.,* 2017). Briefly, RK-13 cells were infected with the vToH-BAC virus at the MOI of 3–5 for 5 h. The cell pellet was washed with PBS and resuspended in DNA extraction buffer (10 mM Tris, pH 8; 10 mM EDTA, 0.6% SDS and 10 mg/ml

of RNAse). Then 10 µl of proteinase K (10 mg/ml) was added and incubated at 56°C for 60 min. After that 250 µl of 5 M NaCl was added and incubated overnight at 4°C. Subsequently, the supernatant was collected after centrifugation at 18,000 × *g* and DNA was purified by phenol-chloroform extraction followed by precipitation of DNA using 2.5 vol. of ethanol. Subsequently, 1–5 µg of the purified DNA was electroporated into DH10B *E. coli* (Shizuya *et al.,* 1992; Messerle *et al.,* 1997) and selected against chloramphenicol resistance. The plasmid DNA obtained from the positive clones was confirmed by restriction enzyme digestion analysis and named as pToH-BAC.

The recombinant virus (vToH-BAC) was regenerated by transfecting the 5 µg of pToH-BAC plasmid into RK13 cells using PEI as described above. The infected cells and supernatants were collected 48 h after transfection, freeze-thawed, and a virus stock named vToH-BAC was prepared.

Generation of gE gene deletion mutant virus (vToH-BACΔgE) from pToH-BAC DNA construct. Deletion of gE gene (ORF74) from vToH genome were carried out using pToH-BAC construct employing En Passant-based two-step Red recombination mutagenesis strategies as described by Tischer *et al.* (2006). A positive selection kanamycin marker cassette (KAN cassette) was prepared by amplifying the kanamycin coding region from pLAY2 vector using designed primers (gE-Forward: 5'-CAAT GTTTACATTATTTGTGATTGGGACGGTCCACTGGGAGGTG GAAAATGAGCAGCAACAGCTAGGGATAACAGGGTAATCG-3' and gE-Reverse: 5'-GACTCCCCCGAAGCACTCTGTGTTATCGCTGTT GCTGCTCATTTTCCACCTCCCAGTGGACCGGCCAGTGTTACAAC CAATTAACC-3') having 45 bp sequences homologous to both flanking sites of the gE gene and an I-SceI restriction enzyme site. The product was purified and confirmed by gel electrophoresis. For mutagenesis, the pToH-BAC plasmid was transfected into *E. coli* strain GS1783 followed by electroporation of GS1783 cells with KAN cassette as per standard protocol. The colonies were picked up from LB agar plates containing chloramphenicol (CAM) and kanamycin (KAN) and subjected to colony PCR for confirmation. Subsequently, the 1st red mutant construct- pToH-BAC-KAN was confirmed by *HindIII* restriction enzyme digestion profile of DNA and confirmed clones were subjected to 2nd Red recombination reaction to remove inserted KAN cassette. The gE gene-deleted clones were selected by replica-plating and plasmids purified from confirmed clones were analyzed by restriction enzyme digestion of the DNA.

The pToH-BACΔgE DNA (2 µg) was transfected in RK 13 cells using polyethyleneimine (PEI) cationic polymer compound (PEI:DNA @ 1:2), incubated at 37°C and observed for viral plaques with green fluorescence. The regenerated virus was adapted into RK13 cell cultures and analyzed by restriction enzyme digestion profiling of DNA. Subsequently, the g71 gene was restored in the mutant virus by replacing the miniF sequences through homologous recombination with the complete g71 gene amplicon in RK13 cells. The *gfp*-negative mutant virus was designated as vToHΔgE.

Restriction enzyme digestion analysis of plasmid and viral DNAs. The plasmid DNA from clones (pToH-BAC & pToH-BACΔgE) was purified using Qiagen Maxi Plasmid Kit (Qiagen, USA) as per the manufacturer's instruction. The DNA of different viruses (vToH, vToH-BAC and vToH-BACΔgE) was extracted from supernatants of infected RK-13 cells after complete CPE using Qiagen Viral RNA kit (Qiagen, USA). One microgram of the purified DNA from plasmids and viruses was digested with *Hind*III restriction enzyme for 1 h at 37°C and electrophoresis was carried out in 0.8% agarose to generate the profile of DNA fragments by applying 45 V for 20 h. After running, the gel was stained with ethidium–bromide and DNA fragment profile was obtained.

Estimation of plaque size and in vitro growth kinetics of vToH and vToHΔgE. The comparative *in vitro* growth properties of the vToHΔgE virus with wild-type virus (vToH) were evaluated by standard plaque assay through measurement of the plaque area and growth kinetics.

Plaque size measurements. Confluent monolayers of RK13 cells were infected with 20 PFU of the respective viruses. After 1 h of incubation, the cells were over-layered with a semi-solid medium containing 1.5% low viscosity carboxymethyl cellulose (Sigma-Aldrich). The plaque sizes were analyzed 2 days post-infection (dpi) by visualization under the microscope upon formalin-fixing and staining with 0.2% crystal violet. Fifty plaques were photographed and plaque areas were determined using the ImageJ software (http://rsb.info.nih.gov/ij/). Values were calculated and compared to those for plaque areas induced by wild-type viruses, which were set to 100%.

Estimation of growth kinetics. RK13 cells were infected with respective viruses (MOI of 0.01) and incubated for different time points (6, 12, 24, 36, 48 and 72 h) after removing the unbound viruses. The cells and supernatant were collected and subjected to the calculation of viral titres by standard plaque assays. The serial 10-fold dilutions of collected supernatant and cells after freeze-thawed were added in triplicate onto confluent RK13 cells. The titers of the viruses at each time point were calculated from each dilution of both supernatant and cell lysate separately. The growth curves were determined in three independent experiments for both supernatant and cell lysate. Mean and standard deviations were calculated and plotted. Student's *t*-test was used to evaluate the differences in viral growth kinetics .

In vitro evaluation of host tropism of mutant EHV1 virus (vToH-BACΔgE). The host tropism of the mutant vToH-BACΔgE virus was investigated in cell lines from different host species including RK13 (Rabbit kidney cell), Vero cells, Equine lung cells (EL), Madin-Darby Bovine Kidney (MDBK) and PK-15 (Porcine Kidney line) cell monolayer as described earlier (Spiesschaert *et al.,* 2015). The monolayer of cell culture was prepared in the 6 well plates with 10% MEM and infected at MOI of 0.01 with the mutant virus. The characteristics of CPE were recorded on the third day.

Animal experiments. The *in-vivo* experiment in BALB/c mice was carried out after obtaining necessary approval from Institute Animal Ethical Committee (NRCE/IAEC/ dated:18.09.2017&NRCE/ CPSCEA/2018-19, 7/2/2019) and Institute Biosafety Committee (NRCE/IBSC/date:13.08.2018). The BALB/c mice (3–4 weeks old) were inoculated intra-nasally with wild-type EHV1 (vToH) and mutant virus (vToHΔgE) at the dose of 1×10^5 PFU in PBS (20 µl) under the mild anesthesia (Ketamine + Xylazine). Mice in each group were observed daily for clinical signs such as hunching in corners, crouching, ruffled fur and other signs. Body weights were recorded for all mice on daily basis throughout the period of experiment (14 dpi). The samples (blood, nasal washings and lungs) were collected immediately after euthanizing animals as per the standard procedure.

Gross and histopathological examination. Necropsy was carried out on all animals meticulously and gross lesions were recorded. The lungs from the necropsied animals were collected and immersion fixed in 10% buffered formalin for histopathology. For histopathological analysis, formalin-fixed tissues were processed by conventional method as per protocol described by Luna *et al.* (1968). Briefly, the tissues were processed for making paraffin-embedded blocks and thin sections (4–5 µm) were cut on microtome (Leica RM9000). The sections were picked on Poly-L-Lysine (0.01%) coated glass slides, stained with Hematoxylin and Eosin and mounted with coverslip using DPX stain. The stained sections were visualized under a microscope (Nikon TI2-S-HU) and histopathological lesions were recorded.

Scoring of gross and histopathological lesions. Gross lesion scoring (maximum lesion score of 5) for the lungs was performed based on the following criteria – congestion (+1 grade); red hepatization (+1-3 grades); grey hepatization (+1 grade). The grading of histopathological lesions at various intervals was based on the extent of severity of lesions as per the criteria previously followed by Pavulraj *et al.* (2015) with some modifications. The following criteria were considered on a scale of 0 to 5 and included – neutrophil/macrophage/lymphocytic infiltration in the interstitium; interstitial consolidation, edema and thickening of the interlobular septa; perivascular /peribronchiolar lymphocyte infiltration; desquamation and necrosis of the bronchial and bronchiolar epithelium.

Statistical analysis. Suitable statistical test(s) were implemented using GraphPad PRISM® software version 8.1 (https://www.graphpad.com) to test the significance of differences in viral plaque sizes and viral replication kinetics.

Results

Generation of EHV1-BAC clone (pToH-BAC)

For the construction of a BAC clone of EHV-1 strain Tohana, the mini-F plasmid (8874 bp) carrying sequences of *gfp* and *xgpt* genes was inserted into the EHV1 genome by replacing the 1568 bp region of gp2 (ORF71) gene by homologous recombination in RK13 cells. After the development of complete CPE, the virus progeny was titrated in RK13 cells and virus plaque showing green fluorescence was picked up. A homogenous fluorescence of vToH-BAC virus stock was obtained after six rounds of purification of fluorescent plaques. The circular intermediates of the recombinant viral genome were isolated from infected RK13 cells, electroporated into *E. coli* DH10B and CAM resistant colonies were selected. The BAC DNA termed pToH-BAC was purified from CAM resistant bacterial colonies and characterized by *Hind*III *restriction enzyme* digestions (Fig. 1a). The banding pattern was compared with the *in silico* predicted DNA pattern after insertion of mini-F plasmid and also with the wild-type vToH virus. The inserted 8874 bp of mini F plasmid lacking *Hind*III restriction enzyme site leads to the combining of two bands of 9675 bp and 1108 bp in the wild-type virus into a single band at 18088 bp in pToH-BAC (Fig. 1a). The 7306 bp difference was due to the insertion of 8874 bp mini F plasmid in place of 1568 bp in gp2 gene. The results confirmed that mini-F plasmid was successfully inserted into the EHV-1 genome, which could be maintained and propagated in *E. coli*.

Regeneration of infectious EHV1-BAC virus (vToH-BAC) from BAC clone

The infectious recombinant EHV1 was reconstituted successfully upon transfection of the purified pToH-BAC DNA into RK13 cells. The cells expressing GFP were observed and foci of viral plaques were obtained on day one after transfection. The viral plaques were visible on the third day and complete CPE had developed by day five after transfection. The regenerated virus was further confirmed by restriction enzyme digestion analysis of the reconstituted virus DNA digested with *Hind*III. The banding pattern of the linear reconstituted virus was similar to the pattern of the wild-type vToH virus (Fig. 1a), with the exception of larger 18088 bp band due to the insertion of the mini-F sequences. The circular pToH-BAC and regenerated linear virus differ in restriction enzyme digestion profile with 7392 bp and 6208 bp band pattern, respectively.

Construction of gE gene-deleted mutant EHV1 (vToHΔgE) virus

The gE gene was successfully deleted from the pToH-BAC construct by En Passant mutagenesis. The pToH-BACΔgE construct was confirmed by comparing the *Hind*III restriction enzyme digestion pattern with

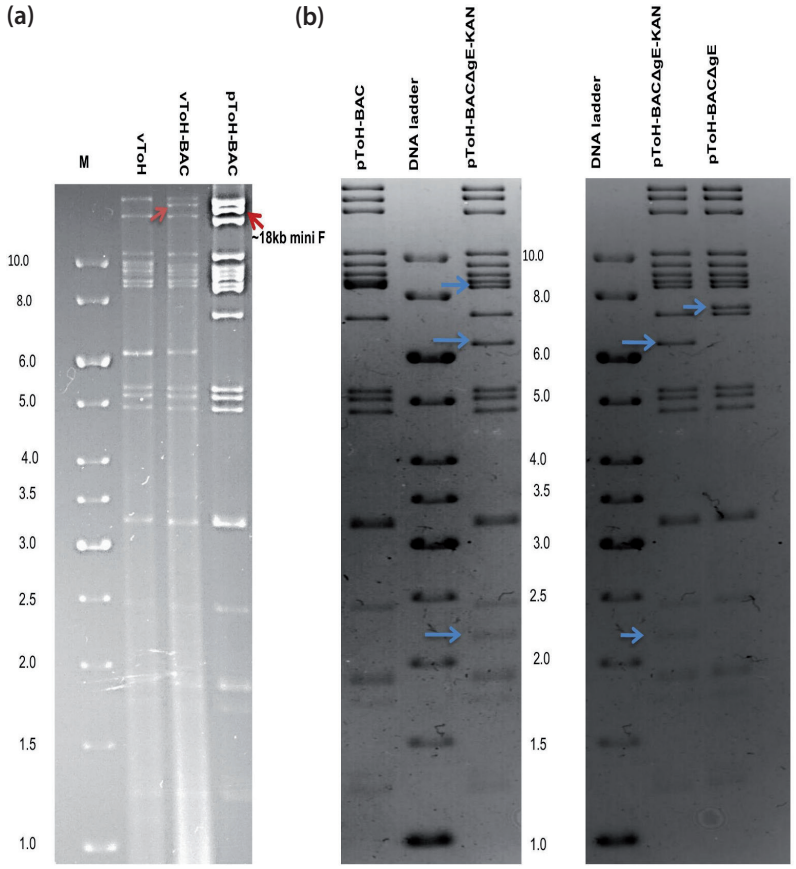

Fig. 1

The *Hind*III restriction enzyme digested DNA fragment profiling for confirmation of clones and mutant EHV1
(a) DNA fragment profile of the genomes of wild-type EHV1 virus (vToH), pToH-BAC and regenerated virus (vToH-BAC). The inserted mini-F plasmid is marked with an arrow. **(b)** Confirmation of gE gene-deleted EHV1 mutant construct (pToH-BACΔgE) by *Hind*III restriction enzyme digestion profiling. The expected band changes are depicted with arrow marks.

pToH-BAC. The two bands of 6399 bp and 2181 bp were observed in 1st red clones instead of a single band of 9303 bp in pToH-BAC construct (Fig. 1b). The banding pattern varied due to the insertion of 1024 bp KAN cassette replacing 1747 bp region (1646 bp of gE gene and 101 bp of upstream region). The 2nd red clones were confirmed by amplification of the expected 313 bp region due to the deletion of the gE gene. The clones were further confirmed by matching the *Hind*III digested fragment pattern, which showed a 7556 bp band in 2nd red clones instead of a 9303 bp band in pToH-BAC (Fig. 1b). The difference of 1747 bp in band pattern indicates the deletion of the targeted region of the gE gene. The gE gene deleted mutant construct was designated as pToH-BACΔgE. Subsequently, the g71 gene was reconstituted in the mutant virus by replacing the mini-F sequences through homologous recombination in RK13 cells. The gfp-negative gE gene deletion mutant virus - vToHΔgE was propagated in RK13 cells.

In vitro studies

Evaluation of plaque sizes and growth properties of reconstituted vToHΔgE virus

The plaque sizes and the growth kinetics of the mutant EHV1 (vToHΔgE) in RK13 cell line were compared with the wild-type EHV-1 strain Tohana (vToH) to determine the effect of deletion of the gE gene. The average plaque size of the deletion mutant virus was evaluated at 48 h post-infection (pi) in RK13 cells. The mean plaque areas recorded were 61.6±0.22 and 18.3±0.07 mm^2 in vToH and vToHΔgE, respectively (Fig. 2a,b). A significant difference ($p > 0.0001$; Kruskal-Wallis test) in plaque areas (~70.29 %) was observed in vToHΔgE compared to the wild-type vToH.

The replication of the vToHΔgE virus was assessed by measuring the extracellular and intracellular virus titers in RK13 cells by one-step growth kinetics assay. The

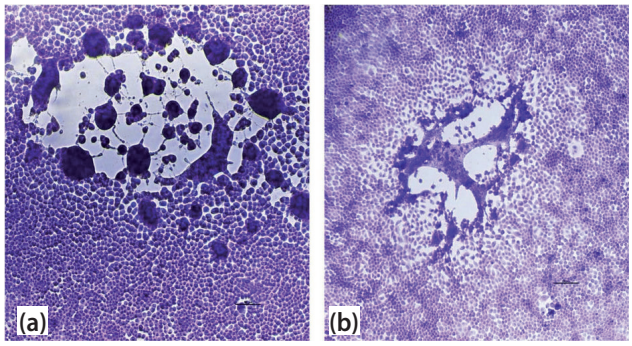

Fig. 2

Comparative plaque morphology of vToH and vToHΔgE
Plaque sizes of both viruses in RK13 cells were measured on 3rd day (x100). **(a)** plaque size of wild EHV-1 (vToH) virus, **(b)** plaque size of gE gene deleted mutant EHV1 (vToHΔgE) virus.

titers of both mutant and wild viruses peaked at 48 h p.i. followed by a decline at 72 h p.i. The replication of mutant viruses was almost identical to wild-type virus without any significant difference (Fig. 3a,b). The extracellular and intracellular viral titers of vToH were 4.7 to 4.8×10^6 pfu/ml and 5.5 to 5.9×10^5 pfu/ml, respectively; whereas titers of the mutant virus were 5.5 to 5.7×10^6 pfu/ml and 5.2 to 5.8×10^5 pfu/ml. Overall, the extracellular viral titers of both vToH as well as the mutant virus were approximately 1.0 log higher than the intracellular titers at 36–48 h. The extracellular titer of the mutant virus was around 0.2 log higher than the vToH from 12 h onwards. However, the intracellular titers of the deletion mutant were around 0.2 log lower than the wild-type virus (vToH). There was no significant difference among any of the groups at all intervals.

Evaluation of the host tropism of mutant EHV1 virus

The spectrum of host tropism of the mutant vToH-BACΔgE virus was evaluated in different cell lines to adjudge their use as vector vaccine against different pathogens of various host species. The mutant virus is capable of replication in all tested cell lines (Fig. 4). The mutant virus showed a comparatively higher percentage of CPE in RK13 followed by MDBK, EL, Vero and PK15 cells.

In vivo studies in murine model

Body weight percentage changes in mice infected with vToHΔgE in comparison to vToH

The mutant virus vToHΔgE caused significantly less weight loss compared to wild-type vToH (Fig. 5). The weight reduction was lowest on day 3 dpi. The weight gain of the animals started after 3 dpi and reached to their original weight on 6 dpi in vToHΔgE group.

Evaluation of gross pathological lesions in lungs of mice infected with vToH and vToHΔgE

The gross pathological lesions in infected mice were restricted to respiratory tract, particularly in lungs as the virus primarily replicates in lungs in the mouse model. The lesions including congestion and red hepatization were observed until 3 dpi, followed by gray hepatization initiated at hilus region and progressed to cover entire lungs in wild virus group with progression of days. The lesions regressed from 7 dpi onwards and were observed until 14 dpi in this group. The lesions at 3 dpi

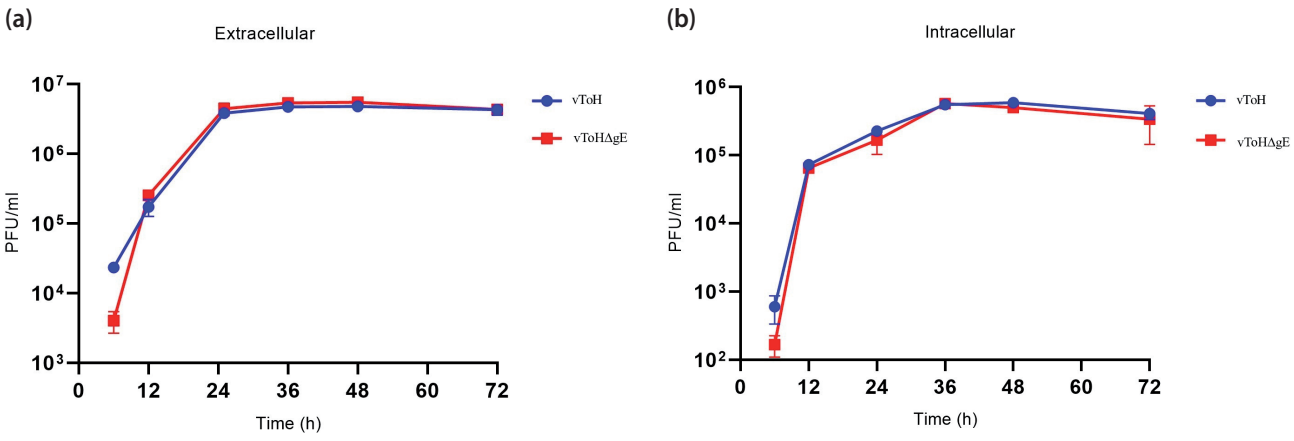

Fig. 3

Growth kinetics of vToH and vToHΔgE in RK13 cell line
(a) Comparative extracellular virus growth pattern. **(b)** Comparative intracellular virus growth pattern.

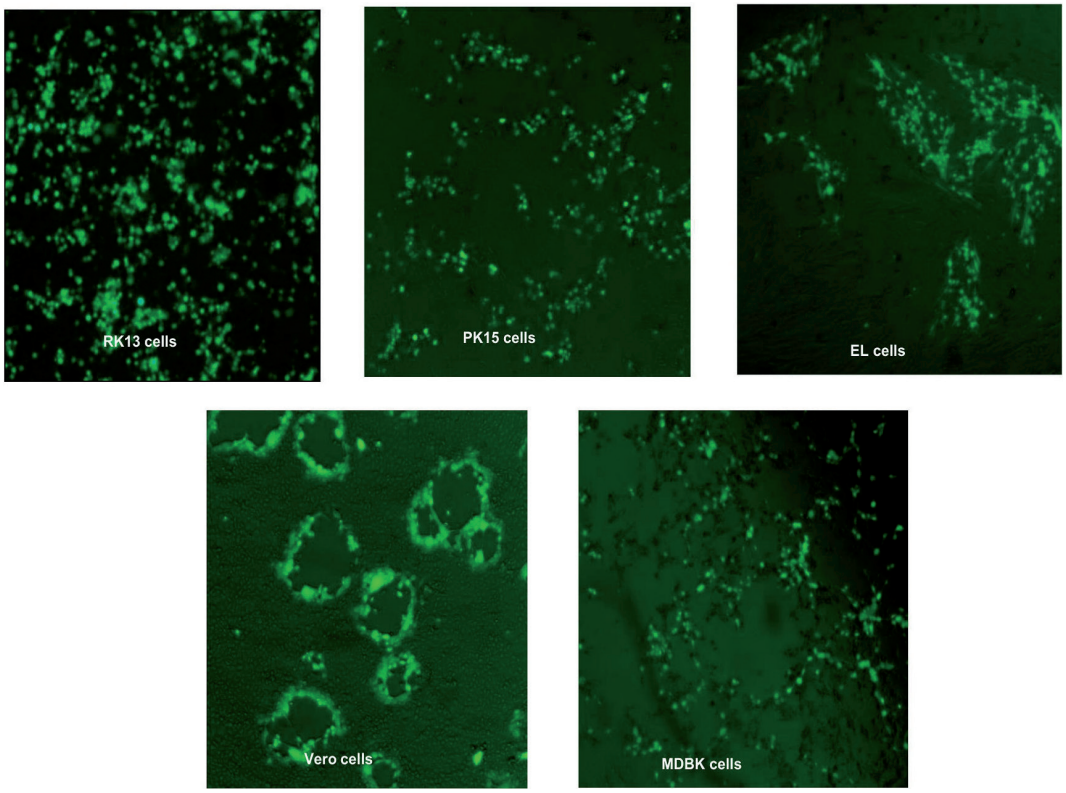

Fig. 4

Evaluation of host tropism of vToHΔgE virus
The vToHΔgE virus grows in rabbit kidney cells (RK13 cell line), porcine kidney cells (PK15 cell line), equine fetal lung cells (EL cells), Vero cells and MDBK cells.

in vToH and vToHΔgE group mice included congestion, which was milder in intensity and focal in the vToHΔgE group (Fig. 6a,b). At 5 dpi, the lesions changed from red hepatization to focal gray hepatization (Fig. 6c,d). The intensity of lesions was decreased in both groups, however, the severity was lower in the vToHΔgE group. At 7 dpi, the lesions were mainly the gray hepatization with focal distribution in the vToH mice, whereas no lesion was observed in the vToHΔgE group mice (Fig. 6e,f). At 14 dpi, there were no lesions in the lungs of mice from both experimental groups. Overall, the gross lesion score and the lesion severity were lower in the vToHΔgE group compared to the vToH group.

Histopathological changes in lungs of mice infected with vToH and vToHΔgE

The histopathological changes in sections of lungs of mice from all experimental groups were investigated and lesions were scored. The pneumonic changes in the wild virus group included alveolitis, necrotizing bronchitis and bronchiolitis with infiltration of neutrophils and lymphocytes at 1 dpi, followed by infiltration largely comprising lymphocytes, macrophages with peribronchial and perivascular cuffing from 3 dpi onwards. The lesions including cellular infiltration, thickening of alveolar septa, and consolidation of lung parenchyma were widespread and peaked at 5 and 7 dpi with signs of resolution at 14 dpi. The lesions in lungs from the

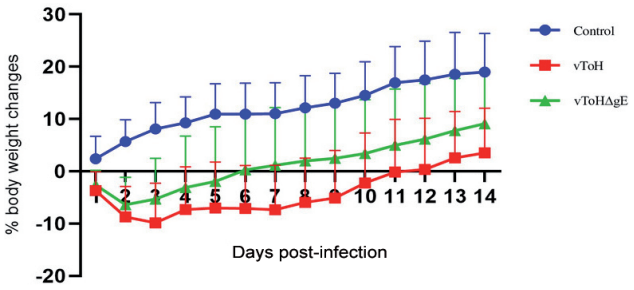

Fig. 5

Body weight percentage changes in mice infected with the vToH and vToHΔgE viruses
Mean body weight loss or gain in mice were compared.

vToHΔgE group mice were similar to those observed in wild virus group but the severity of lesions was moderate at 3 dpi and mild at 5 dpi (Fig. 7a-d). At 5 dpi, the diffuse infiltration of inflammatory cells was observed in both groups. From 7 dpi onwards, lesions were negligible in vToHΔgE groups whereas moderate infiltration was observed in the wild virus group (Fig. 7e,f). The overall scoring of histopathological lesions showed significant reduction in lesions in vToHΔgE in comparison to the wild-type virus group.

Discussion

The BAC clones of various herpesviruses have been constructed and exploited for application in studying gene function, pathogenesis and development of live modified vaccines. In the present study, we have successfully generated an infectious BAC clone of indigenous virulent EHV-1 strain Tohana by inserting the mini-F plasmid sequence replacing the gene 71, which encodes glycoprotein gp2 essential for maturation and egress of

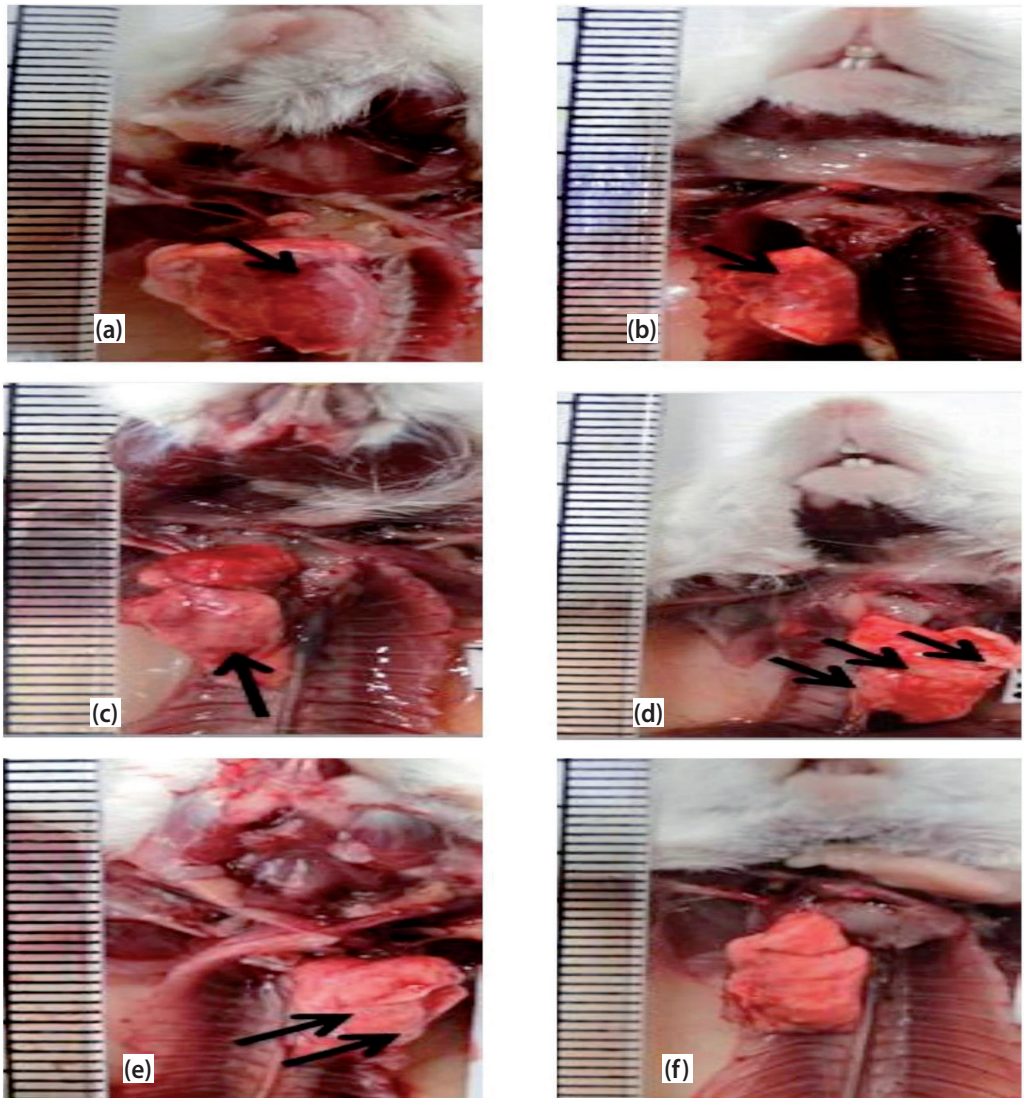

Fig. 6

Gross pathological changes in lungs of mice infected with the vToH and vToHΔgE viruses

(a) Diffuse congestion on the dorsal surface of the left lung in vToH-infected mice at 3 dpi. **(b)** Focal area of congestion on the cranial lobe of the right lung in vToHΔgE-infected mice at 3 dpi. **(c)** Severe diffused grey hepatization on the dorsal surface of the caudal lobe of the left lung lesion in vToH-infected mice at 5 dpi. **(c)** Focal grey areas with mild congestion on the dorsal surface of the left lung in vToHΔgE-infected mice at 5 dpi. **(d)** Focal grey areas on the dorsal surface of the middle lobe of right lung in vToH-infected mice at 7 dpi. **(e)** apparently normal lung in vToHΔgE-infected mice at 7 dpi.

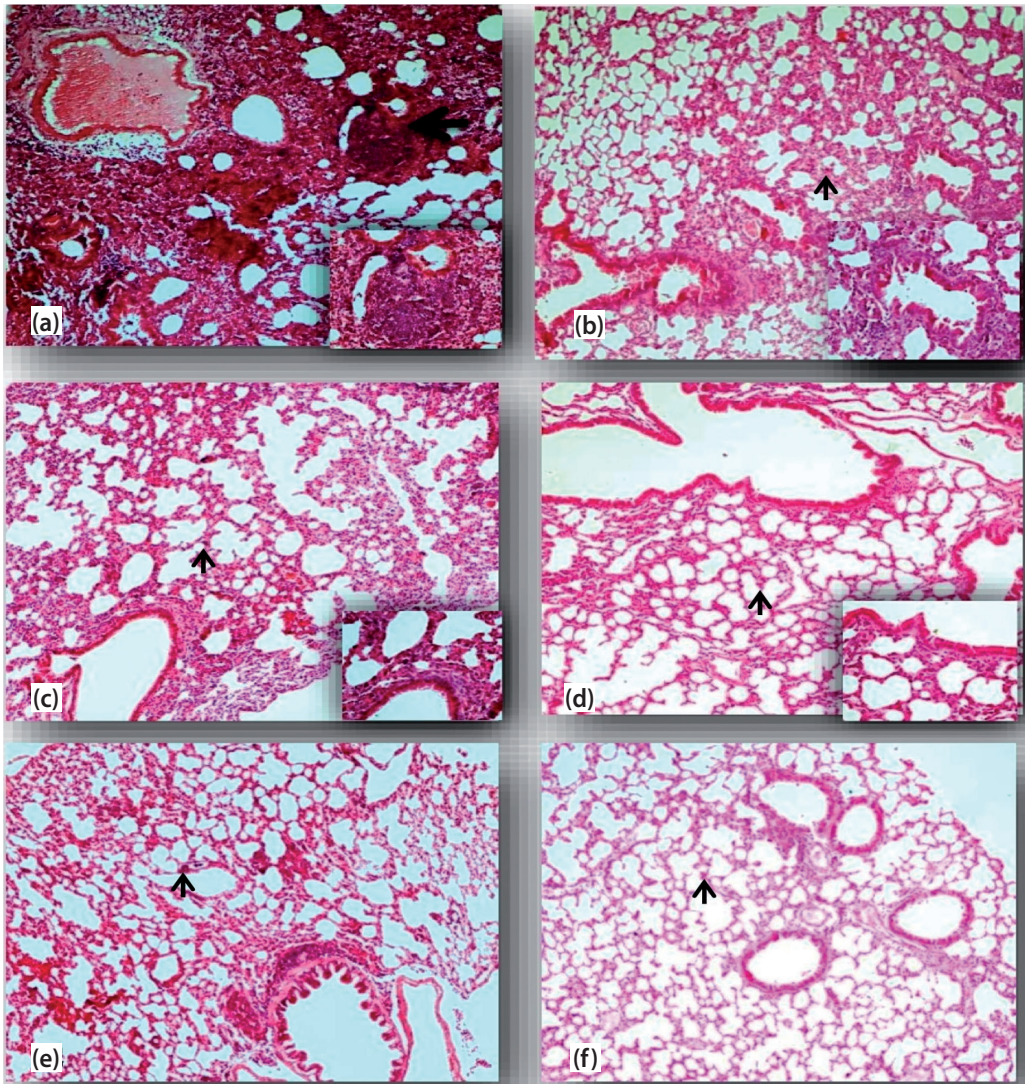

Fig. 7

Histopathological lesions in lungs of mice infected with vToH and vToHΔgE (x100)

(a) Severe congestion, perivascular edema and diffuse interstitial infiltration of MNCs along with multifocal necrosis of bronchiolar epithelium in vToH-infected mice at 3 dpi. **(b)** Moderate interstitial infiltration of mononuclear cells with focal necrosis and hyperplastic changes in bronchiolar epithelium in vToHΔgE-infected mice at 3 dpi. **(c)** Diffused moderate infiltration of lymphocytes and macrophages with moderate necrosis of bronchiolar epithelium in vToH-infected mice at 5 dpi. **(d)** Mild infiltration of lymphocytes and macrophages with focal areas of sloughing (arrow) of bronchiolar epithelium in vToHΔgE-infected mice at 5 dpi. **(e)** Diffused infiltration of lymphocytes and macrophages (arrow) with moderate peribronchial cuffing of lymphocytes and emphysema in lung parenchyma in vToH-infected mice at 7 dpi. **(f)** Normal lung parenchyma with areas of emphysema in vToHΔgE-infected mice at 7 dpi.

the virus, but non-essential for virus replication (Sun *et al.*, 1996). The intermediate circular forms of recombinant EHV-1 virus-containing mini-F were isolated, transfected into *E. coli* and the resulting BAC plasmid showed the expected complete genome sequence of EHV-1 strain Tohana when compared by restriction enzyme analysis with the wild-type virus. The gp2 gene-deleted EHV-1 viruses were reconstituted by transfecting RK13 cells with the BAC plasmid. In the evaluation of growth properties, we observed non-significant differences in replication rates between BAC-derived and wild-type viruses. The reconstituted virus showed a reduction in plaque area compared to the wild-type virus. This is probably due to lower binding capacity and impairment in the maturation and egress of the virus deficient in gp2 (Sun *et al.*, 1996; Rudolph *et al.*, 2002; Smith *et al.*, 2005). Smith *et al.* (2005) demonstrated the critical role of full-length gp2 in the induction of severe respiratory immunopathology in CBA

mice. Titration of the culture supernatant and cell lysates from infected RK13 cells showed that the EHV1-BAC clones produced cell-free viruses. However, the titers of cell-free viruses were lower than those produced by wild vToH virus. The results corroborate with the previous findings, which showed that the gp2 gene plays an important role in virus egress after secondary envelopment (Sun *et al*., 1996; Rudolph *et al.,* 2002).

The BAC clone was developed to maintain large viral genome stably in *E. coli* with low and stringently controlled copy number, and the EHV1-BAC-derived viruses could be propagated several times *in vitro*. Previous studies had shown that herpesvirus BAC genomes propagated in *E. coli* DH10B (a *rec A* gene mutation strain) are genetically stable due to minimized recombination events in this *E. coli* strain (Lee *et al.,* 2001). However, recombination events in BAC DNA have also been reported (Adler *et al*., 2000). To evaluate the stability issue of the pEHV1-BAC plasmid, *E. coli* harboring the BAC was passaged several times in LB broth containing chloramphenicol and on LB-CAM plates, followed by isolation of BAC DNA from overnight cultures of single bacterial colonies and analysis by restriction endonuclease digestion. We found that the *Hind*III profiles of passaged BAC DNA were identical to that of the original BAC, suggesting that the EHV1-BAC plasmid is stable in *E. coli* upon multiple passages.

After successful construction of the EHV1-BAC clone, its utility has been assessed by generating the gE gene deletion mutant virus by manipulating the generated EHV-1 BAC genome. We have targeted the gE gene because the encoded glycoprotein is associated with the virulence of the virus (Rudolph, 2002). Further, the functional analysis of the homologous glycoproteins of other viruses *viz.,* HSV-1, pseudorabies (PRV) and varicella zoster virus (VZV) reported earlier showed that gE protein form a non-covalent complex together with gI protein and the complex binds with immunoglobulin G (IgG) Fc protein, leading to cell-to-cell spread of the virus (Johnson and Huber, 2002). Thus, the gE and other glycoproteins encoded by genes of *Herpesviruses* have been exploited for attenuation of the virus and subsequent use as live vaccine production (van Engelenburg *et al.,* 1994; Jacobs and Kimman, 1994; Kaashoek *et al*., 1995; Tsujimura *et al.,* 2009). These reports described that the deletion of the gE gene causes reduction of the plaque diameter of the mutant virus. We also observed around 70% reduction in plaque size of the gE mutant EHV1. However, the evaluation of the growth kinetics of the mutant EHV1 revealed that the deletion of gp2 and gE genes has low effect on the overall replication of the virus. The results further corroborate with the previous reports showing that the gE gene deletion in other alphaherpesvirus genomes causes distinct plaque morphologies (Matsumura *et al.,* 1998; Damiani *et al.,* 2000; Wisner *et al.,* 2000; Trapp *et al.,* 2003).

The *in vitro* analysis of the gE mutant EHV1 suggests the proper attenuation of the virus, which may be used as a good vaccine candidate for developing a marker vaccine to control EHV-1 infection in equines. To adjudge the efficient attenuation of the mutant virus, *in vivo* evaluation of the generated mutant was carried out in a well-established mouse model. The mice inoculated with deletion mutant virus (vToHΔgE) showed significantly less bodyweight loss, lower gross lung lesion score and lesion severity and lower overall score of histopathology lesions in lungs compared to vToH. The overall findings of the *in vivo* analysis indicate that the gE mutant EHV1 is sufficiently attenuated.

We also assessed the replication of the gE-deleted bacmid-derived virus (vToH-BACΔgE) in different host cells. This indicates that EHV1-BAC virus was efficiently transduced in the cells and the transgene (*gfp*) was expressed efficiently. This suggests that the developed EHV1-BAC construct may be used for the delivery of immunogen from other pathogens for developing the vectored vaccines in different animal species.

In conclusion, we have successfully generated the infectious bacterial artificial chromosome clone of a virulent Indian strain of EHV1, which will provide basis for quick and efficient mutation of viral genome in a well-established prokaryotic system. The feasibility of mutation of EHV1-BAC was proven by constructing the gene deletion mutant EHV1 virus and its pathological evaluation. The successful replication of recombinant EHV1 viruses and efficient attenuation demonstrates its potential for the development of modified live vaccine for control of EHV1 besides its usage as a vector for other vaccines.

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