Us3 and Us9 proteins contribute to the stromal invasion of bovine herpesvirus 1 in the respiratory mucosa

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Abstract

Bovine herpesvirus 1 (BHV-1) infection may lead to conjunctivitis, upper respiratory tract problems, pneumonia, genital disorders and abortion. BHV-1 is able to spread quickly in a plaque-wise manner and invade by breaching the basement membrane (BM) barrier in the respiratory mucosa. BHV-1 Us3, a serine/threonine kinase, induces a dramatic cytoskeletal reorganization and BHV-1 Us9, a tail-anchored membrane protein, is required for axonal transport of viruses in neurons. In this study, we investigated the role of Us3 and Us9 during BHV-1 infection in the respiratory mucosa. First, we constructed and characterized BHV-1 Us3 null, Us9 null and revertant viruses. Then, we analysed the viral replication and plaque size (latitude) in Madin–Darby bovine kidney (MDBK) cells and the respiratory mucosa as well as viral penetration depth underneath the BM of the respiratory mucosa when inoculated with these recombinant viruses. Knockout of Us3 resulted in a 1 log_{10} reduction in viral titre and plaque size (latitude) in MDBK cells and the trachea mucosa. There were no defects in the cell-to-cell spread observed for BHV-1 Us9 null virus. Both BHV-1 Us3 null and Us9 null viruses showed a significant reduction of plaque penetration underneath the BM; however, penetration was not completely inhibited. In conclusion, the current findings demonstrated that Us3 and Us9 play an important role in the invasion of BHV-1 through the BM of the respiratory mucosa, which shows the way forward for research-based attenuation of viruses in order to make safer and better-performing vaccines.

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is a member of the Alphaherpesviridae. BHV-1 infection may cause conjunctivitis, upper respiratory tract problems, pneumonia, genital disorders and abortion [1–3]. The BHV-1 virion is composed of a capsid containing a double-stranded DNA genome, which is surrounded by a tegument layer and an envelope containing viral glycoproteins.

Alphaherpesviruses of different species have evolved in various ways to reach deeper tissues of the upper respiratory tract for further spread and inducing latency. In our laboratory, using ex vivo trachea mucosa explants, it was found that BHV-1 is able to disseminate quickly in a plaque-wise manner and invade in lamina propria by breaching the basement membrane (BM) barrier [4]. BHV-1 Us3 can alter intracellular localization of the virus and has been found to colocalize with microtubules in cell protrusions, which implicates possible direct interactions of Us3 with kinesin or dynein motors [5]. There is some evidence that Us9 and UL56 together with kinesins might contribute to the directional transport of alphaherpes viral glycoproteins in the epithelial cells of the respiratory mucosa [6].

The Us3 gene, which is highly conserved among alphaherpesviruses, codes for a serine/threonine kinase. It is a multifunctional protein that has been reported to play a role in the nuclear egress of capsids, prevention of apoptosis, modulation of host immune response and rearrangement of the cytoskeleton [7–9]. BHV-1 Us3 is a 58 kD protein, which possesses a protein kinase activity [10]. It has been reported that BHV-1 Us3 can induce morphological changes of the infected cells based on dramatic cytoskeletal reorganization. It is not involved in the inhibition of apoptosis [5].
The Us9 protein is an unglycosylated type II tail-anchored membrane protein that is highly conserved among members of the alphaherpesvirus subfamily [11, 12]. It is essential for axonal sorting of viral structural proteins and localizes predominantly to the trans-Golgi network [12]. BHV-1 Us9 is an early (β)/late (γ1) protein [13]. Us9 protein has one predicted protein kinase C phosphorylation site and four potential casein kinase II phosphorylation sites. It is readily phosphorylated, yielding a protein with a molecular weight of 30–32 kDa [13]. BHV-1 Us9-deleted mutants are not shed from the nose or eyes following dexamethasone-induced reactivation [14].

The gC protein is a late γ2 protein, which requires ongoing DNA replication for its expression [15]. The gC protein is obviously involved in cell-attachment mechanisms [16]. In addition, gC null virus is only slightly reduced in virulence and is not impaired in cell-to-cell spread [17].

The directional transport of BHV-1 towards the BM is important for virus dissemination and invasion to penetrate the BM. As there is no UL56 in the genome of BHV-1, we focused on Us3 and Us9 to examine their role in the invasion of BHV-1 through the BM. The ex vivo trachea mucosa model and Us3, Us9 as well as gC null mutants were used as tools for the identification of the invasion determinants. The gC null mutant was included as a negative control.

RESULTS

Characterization of BHV-1 Us3 null, Us9 null, gC null and their revertant viruses

The recombinant viruses, BHV-1 Us3 null, Us9 null, gC null and revertant viruses were analysed by Western blotting (WB; Fig. 1). Us3 (58 kDa), Us9 (32 kDa) and gC (91 kDa) were successfully deleted and rescued.

Knockout of BHV-1 Us3 resulted in a defect in virus replication in MBDK cell cultures and trachea mucosa explants

The characterization of BHV-1 Us3 null, Us9 null, gC null and their revertant viruses were analysed by Western blotting (WB; Fig. 1). Us3 (58 kDa), Us9 (32 kDa) and gC (91 kDa) were successfully deleted and rescued.
of the Us3 gene fully rescued the viral titres in MDBK cells and trachea mucosa explants. The Us9 null, gC null and revertant viruses did not show any kinetic defect in either MDBK cells or trachea mucosa explants.

The plaque size of Us3 null virus in MDBK cells was reduced by 30.2±5.3 % (P<0.05) at 48 h p.i. (Fig. 2b, c) while in the trachea mucosa the plaque latitude of Us3 null virus was 31.2±6.3 % (P<0.05) smaller compared to BHV-1 WT. The deletion of Us9 or gC resulted in an 8.0±3.5 % (P>0.05) or 6.7±2.3 % (P>0.05) reduction of the plaque size in MDBK cells and 6.1±2.8 % (P>0.05) or 5.7±3.1 % (P>0.05) reduction of the plaque latitude in the trachea mucosa. The restoration of the Us3, Us9 or gC gene resulted in plaque sizes that were similar to those of BHV-1 WT.

**BHV-1 Us3 null or Us9 null viruses induced a severe impairment in stromal invasion in the trachea mucosa**

In a previous study from our laboratory, it was found that the majority of BHV-1 plaques cross the BM at 48 h p.i. [4]. In order to examine the role of Us3 and Us9 in the stromal invasion of BHV-1, the plaque penetration depth underneath the BM was measured for BHV-1 Us3 null or Us9 null viruses (Fig. 3c, d). The plaque penetration of Us3 null virus and Us9 null virus was significantly reduced compared to BHV-1 WT, with 69.1±10.7 % (P<0.01) and 60.2±12.3 % (P<0.01), respectively. The Us3 and Us9 revertant viruses as well as gC null and revertant viruses displayed similar viral penetration characteristics to BHV-1 WT, with no significant differences (P>0.05).

**DISCUSSION**

The respiratory mucosa is an important portal for BHV-1 entry. Previous studies in our laboratory showed that BHV-1 could induce prominent epithelial plaques and penetrate the BM in a plaque-wise manner in the respiratory mucosa. The epithelial plaques displayed a clear stromal invasion at 48 h p.i. [4].

In the present study, we found that the Us3 protein contributes to cell-to-cell spread in both MDBK cells and tracheal mucosa epithelium. Analysis of growth kinetics of Us3 null virus in trachea mucosa explants showed a kinetic defect at 24 h p.i. compared to WT and revertant strains. However, this defect was only observed in MDBK cells starting from 48 h p.i. These defaults were similar to those described for BHV-1 gE/I null virus, which showed a kinetic defect in trachea mucosa explants at 24 h p.i. whereas this defect was only found at 48 h p.i. in MDBK cells [6]. For Us3 null virus, the kinetic defect was also demonstrated by the small plaque size in both MDBK cells and the trachea mucosa. This small plaque size may be related to a hampered rearrangement of the cytoskeleton as BHV-1 Us3 contributes to the formation of cellular projections, which are filled with virus particles [5]. For Pseudorabies virus (PRV), a related alpha herpesvirus, a similar phenomenon was observed. These branched cell...
projections contain both actin and microtubules and may reach several cell diameters in length, which is associated with enhanced virus spread [18]. Whether cellular projections are formed in between epithelial cells of the respiratory tract allowing the virus to spread needs further research. Most of the studies that showed that Us9 protein is involved in cell-to-cell spread are focused on neurons. Deletion of Us9 in PRV is associated with failure of viral spread both in vitro and in vivo [11, 19]. In herpes simplex virus type 1 (HSV-1), it has been found that Us9 plays a critical role in cell-to-cell spread in neurons, but that other viral proteins are involved as well [20]. Similarly, BHV-1 Us9-deleted viruses demonstrated a defective anterograde neuronal spread in neuron culture systems and in vivo [14, 21]. In the present study, it was demonstrated that BHV-1 Us9 displays no defects in cell-to-cell spread in MDBK cells and the respiratory mucosa. PRV Us9 also had no defects in cell-to-cell spread in MDBK cells [11].

More striking was the finding that both Us3 and Us9 proteins contributed to the viral passage across the BM barrier during BHV-1 infection in bovine trachea mucosa explants in our study. Knockout of Us3 or Us9 caused a remarkable reduction of penetration depth underneath the BM in the trachea mucosa at 48 h p.i.; however, penetration was not completely inhibited. For Us3, this defect may be explained by the involvement of serine/threonine kinase Us3-affected signal pathways. Recent work on PRV showed that Us3 protein kinase-mediated invasion in respiratory mucosa explants was hampered by interfering with Rho GTPase signalling [22]. The Us3 protein of HSV-1 has been reported to display an Akt-like activity [23] and Akt has

![Figure 3](attachment://figure3.png)

**Fig. 3.** Viral titres at 2, 24 and 48 h p.i. (a) and plaque latitude as well as plaque penetration at 48 h p.i. (b–d) in trachea mucosa explants inoculated with BHV-1 Us3 null, Us9 null, gC null and their revertant viruses, as well as WT viruses. BHV-1-infected cells are stained green and the BM is stained red. Two-way ANOVA; *P<0.05; **P<0.01.
been reported to contribute to BM passage [24]. Thus, HSV-1 Us3 protein may be involved in BM crossing as well. However, this still needs to be confirmed. Another explanation may be that the Us3 deletion in BHV-1 induced a functional change due to the lack of some Us3 phosphorylated proteins. For BHV-1, it has been reported that Us3 is involved in the phosphorylation of tegument proteins VP8 and VP22 [25, 26]. In line with these findings in BHV-1, HSV Us3 has been shown to modulate different viral proteins at various time points during the course of infection [27–29]. Besides, it has been reported that Us3 proteins of HSV and PRV have anti-apoptotic activities that protect cells from apoptosis during infection [30, 31]. This anti-apoptosis can either facilitate the establishment and maintenance of a persistent viral infection or prolong the survival of lytically infected cells in order to maximize virus production [32]. Hence, the anti-apoptosis may function during the viral penetration of the BM. Whether BHV-1 Us3 possesses anti-apoptotic activity in the respiratory mucosa and whether the anti-apoptosis is involved in BHV-1 stromal invasion will be further determined. In the present study, it was shown that Us9 is also important for BHV-1 invasion in the respiratory mucosa. It has been reported that Us9 proteins of both BHV-1 [33] and PRV [34, 35], together with gE or gE/I, are crucial for efficient anterograde-directed transport of viral particles in neurons. In our study, we found that Us9 knockout had no effect on the expression of gE (Us8) or gE/I (Us8/7). However, the expression of Us9 protein in gE null and gE/I null viruses displayed two bands and one with a higher molecular weight of 34 kDa. With calf intestinal alkaline phosphatase (CIP) treatment, WB showed a similar band pattern of Us9 protein in BHV-1 WT, gE null, gE/I null and revertant viruses (Fig. S1a, available in the online Supplementary Material). These results indicated that the high intensity of Us9 protein in gE null and gE/I null viruses may be due to the overphosphorylation of Us9, which has five predicted phosphorylation sites (Prosite). In other words, gE and gE/I complex may mediate a dephosphorylation of the Us9 protein during BHV-1 infection. This is in line with the explanation that gE or gE/I is required for localization of some viral proteins in the Golgi apparatus where proteins are posttranslationally modified [36] and the deletion of gE or gE/I may interrupt this modification and induce overphosphorylation. In addition, for BHV-1 Us9 protein, besides phosphorylation [13], other posttranslational modifications will also be involved as after CIP treatment the molecular weight of Us9 protein (30 kDa) is still higher than expected (14.7 kDa) (Fig. S1a). In neurons, the expression and localization of Us9 is independent of those of gE; however, there is no report on expression and localization of BHV-1 Us9 in respiratory epithelial cells. An increased expression of Us9 protein was observed in trachea mucosa explants inoculated with gE and gE/I null virus compared to the WT and gE/I revertant viruses (Fig. S1b). These data indicate that some interactions exist between Us9 and gE or gE/I during BHV-1 infection in the respiratory mucosa. This is in agreement with the recent report that gE, gI and Us9 form a trimolecular complex in HSV-infected Vero cells and the interaction between gE and Us9 is specific but transient [37]. A previous study from our laboratory showed that BHV-1 gE/gI are required for BHV-1 invasion and are involved in a basal sorting towards basal domains in basal cells [6]. Based on these findings, we hypothesize that Us9 works together with gE/I in the viral penetration in the respiratory mucosa. Further work is needed to fully reveal other respiratory invasion proteins.

In conclusion, we have shown that BHV-1 Us3 and Us9 proteins play a crucial role in viral passage across the BM barrier during infection of bovine trachea mucosa explants. BHV-1 Us3 protein also contributes to the lateral cell-to-cell spread in both MDBK cell cultures and tracheal mucosa epithelium, while BHV-1 Us9 null virus showed no defects. The current findings on the role of Us3 and Us9 proteins during BHV-1 infection in the trachea mucosa give new insights in the early pathogenesis events of BHV-1 and show a way for research-based attenuation of viruses in order to make safer and better-performing vaccines.

METHODS

Virus strains and cell line

The BHV-1 Cooper strain (Colorado) was propagated and titrated in MDBK cells grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10 % FBS (Gibco), 1 % sodium pyruvate (Gibco), 0.1 mg ml−1 streptomycin (Certa), 100 U ml−1 penicillin (Continental Pharma) and 1 µg ml−1 gentamycin (Gibco).

Antibodies

The rabbit antiserum against Us3 was a kind gift from Sylvia van Drunen-Littel van den Hurk. The goat antiserum against Us9 and the murine monoclonal antibody specific for gC (10B) was kindly provided by Shafiqul I. Chowdhury. The monoclonal antibodies 14B11 (anti-BHV-1 gE) and 2E12 (anti-BHV-1 gE/gI) were gifts from Brigitte Caij (Veterinary and Agrochemical Research Centre, Belgium). Mouse anti-collagen VII antibodies (Sigma) and FITC-labelled goat anti-infectious bovine rhinotracheitis (IBR) polyclonal antiserum (VMRD) were used for the BM and BHV-1 staining, respectively.

Construction of BHV-1 Us3 null, Us9 null, gC null and their revertant viruses by en passant mutagenesis

A previously established BHV-1 Cooper bacterial artificial chromosome (BAC) clone [38], which transformed the BAC plasmid containing the BHV-1 genome into E. coli strain GS1783 (containing a temperature-sensitive red recombination system and a gene encoding the endonuclease I-SceI in its genome), was used for en passant mutagenesis. The GS1783 cells harbouring pBHV-1 (WT BAC) were maintained in Luria–Bertani (LB) medium with 30 µg ml−1 chloramphenicol. The construction of Us3 null, Us9 null, gC null and their revertant viruses was performed by two-step Red recombination as described earlier [39]. The
construction of Us3-deleted and revertant mutants will be given as an example. Firstly, PCR primers (Table 1) were used to amplify the fragment from the plasmid pEP-Kan-S2 to replace Us3 by the aphAI (kanamycin resistance) gene. Secondly, approximately 100 ng DpnI-digested PCR product from the above was incorporated into 50 μl recombinant and electrocompetent pBHV-1 via electroporation. After arabinose induction, several kanamycin-sensitive clones were obtained and screened via restriction fragment length polymorphism (RFLP), PCR and sequencing. This resulted in pBHV-1-Kan-Us3. Secondly, a similar approach was performed as template to generate the fragment with the ORF of Us3 and the aphAI gene. Finally, the authentic Us3 was reintroduced in pBHV-1-Kan-Us3. We used the obtained pull-out Us3 fragment, which still possessed a kanamycin marker within the ORF of Us3, to transform pBHV-1-ΔUs3. After arabinose induction, kanamycin-sensitive clones were screened by RFLP, PCR and sequencing, co-transfected with the plasmid pCAGGS-nls-cre into MDBC cells. At least three rounds of plaque purification were performed to obtain pure BAC-excised clones. In this article, three rounds of plaque purification were performed to obtain pure BAC-excised clones. In this article, reconstitution BAC-excised vBHV-1 and vBHV-1-ΔUs3 are named WT-R and Us3 null virus, respectively. To construct the revertant virus, the aphAI gene was first inserted into the ORF coding for Us3 of the pBHV-1. A similar PCR was performed as described for the construction of mutant alleles using forward and reverse primers (Table 1) and plasmid pEP-Kan-S2 by en passant mutagenesis. This resulted in pBHV-1-ΔKan-Us3. Secondly, a pull-out of the Us3 region out of the WT BAC was performed. To this end, primers were designed (Table 1) and plasmid pEP-Kan-S2 was used as template to generate the fragment and the WT BAC clone was co-transfected with the plasmid pCAGGS-nls-cre into MDBC cells for BAC excision, three times plaque-purified and finally named vBHV-1-ΔUs3-Rev (Us3 revertant virus). The BHV-1 Us9 or gC null and revertant viruses were constructed in the same way.

Characterization of the BHV-1 Us3 null. Us9 null, gC null and their revertant viruses via WB

WB was used to identify the deleted and revertant proteins, respectively. In brief, BHV-1 Us3 null, Us9 null, gC null and their revertant viruses were propagated (second passage) and titrated in MDBC cells [40]. The monolayer of MDBC cells in six-well plates (Nunc) were inoculated with the above viruses

Table 1. Primers used for generating Us9, Us3 and gC null and revertant viruses

<table>
<thead>
<tr>
<th>Genetic engineering</th>
<th>Gene</th>
<th>Primers</th>
<th>Nucleotide sequence</th>
</tr>
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<tbody>
<tr>
<td>Deletion</td>
<td>Us9</td>
<td>Forward</td>
<td>5′-CTCACACAAAGCCTCGTGGCGGC-TGCTTCGAAGGCAATG-TAAATAGCAAGCGGGGCTT-aggatgaagcataaccttag3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-CCGAGGCAATGCGAAGCTCA-GGCGCGGTGCTGCTATTATCATGCCCTTGAAGCA-caaccaatcaacacctgttag-3′</td>
</tr>
<tr>
<td></td>
<td>Us3</td>
<td>Forward</td>
<td>5′-GGGATCCCGGACAGCCAGCCGAGCTGGCGCG-TCTTGGGCAACCCATG-CAACTCTACGCTGGCCGA-aggtgaagcataaccttag3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-CGCGCGGTGCGGCGCGCGCGGAAA-TGTTAGGGTGTCGCTGCAAGGTGCGATCCCAAGCA-caaccaatcaacacctgttag-3′</td>
</tr>
<tr>
<td></td>
<td>gC</td>
<td>Forward</td>
<td>5′-AGAGACCCACCGAGAAGCCATG-GCCGCGGTCCCGGCAATG-TAAATAGCAAGCGGGGCTT-aggatgaagcataaccttag3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-CGCGCGGTGCGGCGCGCGCGGAAA-TGTTAGGGTGTCGCTGCAAGGTGCGATCCCAAGCA-caaccaatcaacacctgttag-3′</td>
</tr>
<tr>
<td>Revertant – Kan insertion</td>
<td>Us9</td>
<td>Forward</td>
<td>5′-CTCACACAAAGCCTCGTGGCGGC-TGCTTCGAAGGCAATG-TAAATAGCAAGCGGGGCTT-aggatgaagcataaccttag3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-CCGAGGCAATGCGAAGCTCA-GGCGCGGTGCTGCTATTATCATGCCCTTGAAGCA-caaccaatcaacacctgttag-3′</td>
</tr>
<tr>
<td></td>
<td>Us3</td>
<td>Forward</td>
<td>5′-GGGATCCCGGACAGCCAGCCGAGCTGGCGCG-TCTTGGGCAACCCATG-CAACTCTACGCTGGCCGA-aggtgaagcataaccttag3′</td>
</tr>
<tr>
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<td>Reverse</td>
<td>5′-CGCGCGGTGCGGCGCGCGCGGAAA-TGTTAGGGTGTCGCTGCAAGGTGCGATCCCAAGCA-caaccaatcaacacctgttag-3′</td>
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<tr>
<td></td>
<td>gC</td>
<td>Forward</td>
<td>5′-AGAGACCCACCGAGAAGCCATG-GCCGCGGTCCCGGCAATG-TAAATAGCAAGCGGGGCTT-aggatgaagcataaccttag3′</td>
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<td>Reverse</td>
<td>5′-CGCGCGGTGCGGCGCGCGCGGAAA-TGTTAGGGTGTCGCTGCAAGGTGCGATCCCAAGCA-caaccaatcaacacctgttag-3′</td>
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Nucleotides in lower case share homology to pEP-kan-S2. Sequences that are underlined are complementary to each other in inverse orientation.

at an m.o.i. of 1. Twenty-four hours later, the supernatant was gently removed and the cells were collected. Afterwards, the cells were mixed with lysis buffer, boiled for 10 min and subjected to SDS-PAGE (12 % gel) using a BioRad Mini Protean 3 system. For WB analysis, proteins were transferred from the SDS-PAGE gel to a PVDF membrane (Membrane Hybond-P; GE Healthcare). The membrane was blocked overnight in 5 % skimmed milk (PBS with 0.1 % Tween-20) at 4 °C. Subsequently, the blot was incubated with the primary antibodies. The rabbit antiserum against US3, goat antiserum against US9 and murine monoclonal antibody specific for gC (10B) were used to analyse the US3 null, US9 null, gC null and revertant viruses, respectively. Goat anti-rabbit IgG (HRP), rabbit anti-goat IgG (HRP) and goat anti-mouse IgG (HRP) were used as secondary antibodies. As a control for loading, total β-actin (Abcam) levels were assessed. Afterwards, visualization was done by enhanced chemiluminescence (ECL; GE Healthcare).

**Bovine respiratory mucosa explants**

Bovine respiratory mucosa explants were collected from three cows, between 2 and 5 years old, at a local slaughterhouse. The blood was collected to determine the BHV-1-specific neutralizing antibody level. Three animals showing a seroneutralizing antibody titre of <2 for BHV-1-specific antibodies via a complement-dependent seroneutralization test were selected for the study. The collected tracheas were transported on ice in PBS supplemented with 10 µg ml\(^{-1}\) gentamicin, 1 mg ml\(^{-1}\) streptomycin, 1000 U ml\(^{-1}\) penicillin, 1 mg ml\(^{-1}\) kanamycin (Sigma) and 5 µg ml\(^{-1}\) fungizone (Bristol-Myers Squibb). Trachea mucosal were stripped from the proximal portion of trachea and divided into small pieces of 50 mm\(^2\). The explants were placed on fine-meshed gauze with the epithelium upwards for culture on an air–liquid interface at 37 °C with 5 % CO\(_2\). The medium used for cultivation contains serum-free medium (50 % DMEM/50 % Ham’s F-12 GlutaMAX) supplemented with 0.1 mg ml\(^{-1}\) streptomycin, 100 U ml\(^{-1}\) penicillin and 1 µg ml\(^{-1}\) gentamycin.

**Analysis of the replication and dissemination of BHV-1 Us3 null, Us9 null and gC null and revertant viruses in MDBK cells and the trachea mucosa**

Confluent MDBK cells were seeded in 24-well culture plates with inserts and inoculated with 1 ml containing 10\(^{5}\) TCID\(_{50}\) of BHV-1 WT, deleted mutants and revertants for 1 h at 37 °C with 5 % CO\(_2\). Cell supernatant was harvested at 2, 24 and 48 h.p.i. for virus titration. Meanwhile, the cells infected with the respective viruses were stained at 48 h.p.i. to observe the nuclei before mounting with glycerin-DABCO. In between each step, three thorough washing steps were included.

**Statistical analysis**

Three independent experiments were performed and the data were presented as means±standard deviations (Givens and Marley). Two-way ANOVA was used to calculate statistical significance among multiple groups. Data were classified: \(P > 0.05\), not significantly different; \(P \leq 0.05\) (*), significantly different; \(P \leq 0.01\) (**), very significantly different.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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